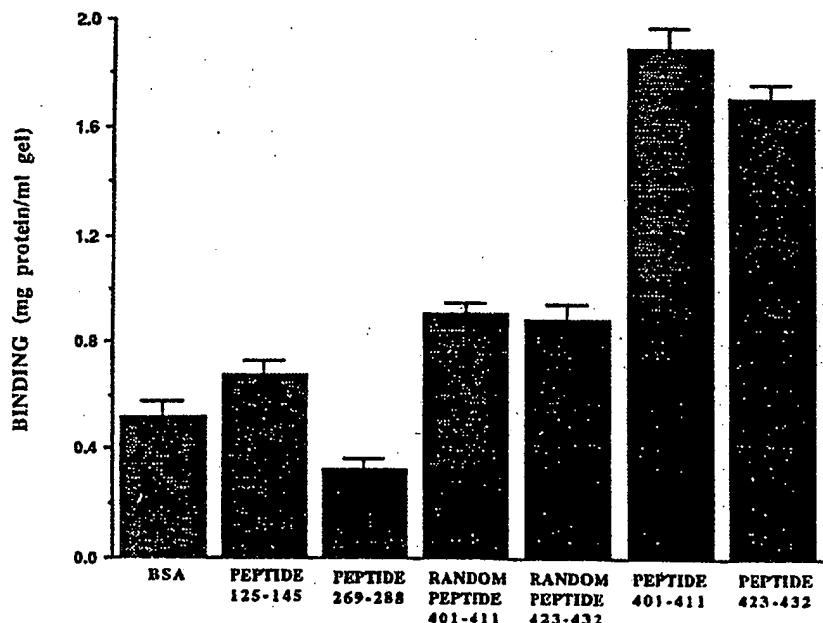




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(57) Abstract

The invention provides a hyaluronan receptor protein involved in cell motility and a DNA sequence encoding the receptor protein. The invention provides novel hyaluronan binding peptides and antibodies to these peptides. Methods are also provided for controlling locomotion and hyaluronan binding in vertebrates.

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HYALURONAN RECEPTOR (RHAMM = RECEPTOR FOR HYALURONAN MEDIATED MOBILITY) AND HYALURONAN BINDING PEPTIDES.

The present invention relates to a novel hyaluronan receptor protein involved in cell motility and to novel hyaluronan binding peptides. It relates also to methods for controlling cell locomotion and for controlling or treating physiological conditions involving cell locomotion.

In the description which follows, references are made to certain literature citations which are listed at the end of the specification.

Background of the Invention

Increased synthesis of hyaluronan (HA) has been associated with the morphogenesis of many tissues (38), and with wound repair, tumour invasion and immune recognition (27,39,40). Further, HA has been proposed to regulate cell locomotion and cytodifferentiation that occur during these phenomena (33,43,44). HA likely mediates these effects either by interacting with specific HA receptors on the cell surface (27,39) or with certain hyaluronan binding proteins in the extracellular milieu. High affinity cell binding sites for HA have previously been demonstrated by kinetic studies on a variety of cell types (27).

A number of cell associated and extracellular HA binding proteins, including link protein (48,47,8), CD44 (89,23,24,73,71,76,79), aggrecan (72), versican (52), GHAP (85), collagen type VI (81) and TSG-6 (51), have been characterized. In link protein, two tandem repeat loops have been found to contain HA binding sites and peptides mimicking specific sequences within these regions have been shown to bind to HA (8).

Prior to the work of the present inventor, however, the amino acid sequence requirements for HA binding had not been recognised.

It has been observed that the transforming oncogene H-ras promotes mammalian cell locomotion (17), although the regulatory mechanisms were unknown. Several observations suggested that these mechanisms are complex 5 and involve, at least, the release of autocrine motility factor(s) (14,20), growth factors (14) and the glycosaminoglycan, hyaluronan (HA) (20,34). In particular, HA appears to function as an autocrine mechanism for stimulating maximal locomotion in ras 10 transformed cells (34). Further, HA is also required for the ability of an autocrine motility stimulating factor, to promote breast carcinoma cell locomotion (20).

HA promoted, ras transformed cell locomotion requires the presence of a novel hyaluronan receptor 15 complex termed HARC (34). This complex of proteins occurs at the cell surface or is released as soluble proteins of MWE 72, 68, 58 and 52 kDa proteins (29). The complex is tightly regulated in vitro (32) and expressed on the leading lamellae and perinuclear region only on 20 rapidly locomoting cells (28,31). Both polyclonal and monoclonal antibodies prepared against this complex block cell locomotion regulated by mutant ras (34).

The nature of the HA receptor involved in cell locomotion had not been described prior to the work of 25 the present inventor.

DESCRIPTION OF DRAWINGS

Certain embodiments of the invention are described, reference being made to the accompanying drawings wherein:

30 Figure 1 shows a restriction map of a 2.9 Kb cDNA clone encoding the HA receptor of the invention.

Figure 2 shows the ultrastructural localisation of the protein encoded by the cDNA of the invention.

35 Figure 3 shows the immunofluorescent localisation of the encoded protein relative to HARC proteins. A : Mab 3T3-5 which specifically reacts with 52-58 kD proteins of HARC. B : Pab to Peptide II.

Figure 4 shows:

Panel A: immunoblots of fractionated proteins probed with Pab to Peptide II as primary antibody.

5 Panel B: immunoblots of fractionated proteins probed with RAS - 10 antibody as primary antibody. Primary antibodies were detected by chemiluminescence. Protein standards are marked by arrowheads and include phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa) and trypsin inhibitor f (21.5 kDa).

10 Panel C: Northern blot of RNA hybridized with a ³²P - labelled 1.4 kb cDNA fragment containing the open reading frame under high stringency.

Panel D: Blots of panel C re-probed with H-GAPDH to control for RNA loading.

15 Figure 5 shows effect of antibodies on HA - promoted locomotion in ras-transformed fibroblast cells.

Figure 6 shows the binding of bacterially expressed RHAMM to biotinylated HA and to antibody to HARC proteins.

20 Lane A: Biotinylated HA added in absence of HA.

Lane B: Biotinylated HA added in presence of 100-fold excess unlabelled HA.

Lane C: control bacterial plasmid with no RHAMM encoding insert.

25 Lane D: Lysate incubated with Mab 3T3-5 to the 58, 52 kDa HARC proteins.

Figure 7 shows:

Panel A: HARC proteins visualised with India ink.

30 Panel B: Immunoblot of HARC proteins reacted with Mab 3T3-5.

Panel C: Immunoblot of HARC proteins reacted with Pab to Peptide I.

Figure 8 shows Western blot assays of GST-RHAMM 35 fusion proteins expressed in E. Coli reacted with:

Lane 2: Pab to a peptide encoded in RHAMM cDNA (peptide ¹²⁵⁻¹⁴⁵)

Lane 4: Mab to RHAMM;

Lane 6: biotin-labelled HA

5 Lanes 1, 3 and 5 show preparations from lysates of E. Coli cells containing the pGEX-2T plasmid without the RHAMM cDNA insert, reacted with the reagents of Lanes 2, 4 and 6 respectively.

Figure 9 shows the truncation strategy for preparing fusion products spanning the RHAMM cDNA.

10 Figure 10A shows visualisation of fusion proteins from truncated RHAMM cDNAs using anti-GST antibodies

Lane 1: HB101 lysate;

Lane 2: fusion protein made from cDNA truncated with AatII + EcoRI;

Lane 3: truncated with BglIII + EcoRI;

15 Lane 4: truncated with NcoI + EcoRI;

Lane 5: truncated with SacI + EcoRI;

Lane 6: untruncated RHAMM cDNA.

Figure 10B shows fusion proteins as in Figure 10A visualised using biotin-labelled HA.

20 Figure 11 shows effect of NaCl concentration on interaction of HA and RHAMM.

Figure 12A shows agarose gel electrophoresis of truncated RHAMM cDNAs:

Lane 1: DNA markers;

25 Lane 2: pGEX-2T containing RHAMM cDNA;

Lane 3: as in 2 but truncated with SacI and EcoRI;

Lane 4: as in 2 but truncated with HphI and EcoRI.

Figure 12B shows transblots of fusion proteins from truncated RHAMM cDNAs binding to biotin-labelled HA:

30 Lane 1: complete RHAMM fusion protein;

Lane 2: fusion protein from RHAMM cDNA truncated with SacI and EcoRI;

Lane 3: fusion protein from RHAMM cDNA truncated with HphI and EcoRI

35 Figure 13A shows competition with HA binding to RHAMM fusion protein by:

Lane 1: BSA (3mg/ml)

Lane 2: peptide ^{aa}401-411 (3mg/ml)

Lane 3: peptide ^{aa}423-432 (3mg/ml)

Figure 13B shows binding of various peptides to HA-Sepharose affinity gel.

5 Figure 14A shows construction of recombinant RHAMM containing HA binding domains, peptide ^{aa}401-411 (Sequence ID No. 3) (□) and peptide ^{aa}423-432 (Sequence ID No. 4) (■). Corresponding nucleotide sequences: Sequence Nos. 20 and 21.

10 Figure 14B shows transblots of GST-RHAMM fusion proteins visualised with Pab to Peptide ^{aa}125-145 (Lanes 1 - 3) or biotin-labelled HA (Lanes 4 - 6):

Lanes 1,4: GST fusion non-recombinant polypeptide ^{aa}1-238;

15 Lanes 2,5: peptide ^{aa}401-411 linked to polypeptide ^{aa}1-238;

Lanes 3,6: peptide ^{aa}423-432 linked to polypeptide ^{aa}1-238.

20 Figure 15 shows effect of RHAMM peptides on locomotion of ras-transformed fibroblast cells.

Figure 16 shows affinity chromatography of synthetic peptides on HA-sepharose.

25 Figure 17A shows the strategy of site-directed mutagenesis of the HA-binding domain, peptide ^{aa}423-432, of RHAMM; Figure 17B shows the resultant amino acid sequences.

Figure 18A shows DNA of HB101 clones containing:

Lane 1: Normal DNA insert for peptide ^{aa}423-432;

Lane 2: DNA insert containing Mutation I;

30 Lane 3: DNA insert containing Mutation II;

Lane 4: DNA insert containing Mutation III;

Lane 5: DNA insert containing Mutation IV;

Lane 6: HB101 with no DNA insert;

35 Figures 18B and C shows Western blots of fusion proteins from clones of 18A, stained with anti-RHAMM antibody (18B) or with biotin-labelled HA (18C).

Figure 19A shows the strategy of aligning link protein peptide^{aa316-325} to RHAMM peptide^{aa1-375} (Sequence ID No. of primer : 15).

Figure 19B shows Western blots of fusion proteins, 5 examined for HA-binding.

Figure 20 shows strategy for aligning peptide^{aa292-300} of CD44 to RHAMM cDNA, nucleotide 1-375, and for mutating the CD44 peptide.

Figure 21:

10 Figure 21A shows Southern blot of PCR products;

Figure 21B shows Southern blot of DNA inserts

Figure 21C shows Western blot of fusion proteins.

15 Figure 22 shows strategy for site-directed mutagenesis of peptide^{aa423-432} (K⁴³⁰, R⁴³¹, and K⁴³² mutated to N⁴³⁰, W⁴³¹ and E⁴³² : Sequence O.D. No. of primer : 19).

Figures 23A, 23B and 23C show the complete nucleotide sequence (Sequence ID No. 1) and the derived amino acid sequence (Sequence ID No. 2) of a 2.9 Kb cDNA clone encoding the HA receptor of the invention.

20 Figure 23A shows nucleotides 1 to 456 of the sequence, and corresponding amino acids. Figure 23B shows nucleotides 457 to 969 of the sequence, and corresponding amino acids. Figure 23C shows nucleotides 970 to 1428 of the sequence and corresponding amino acids.

SUMMARY OF THE INVENTION

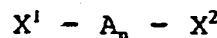
30 The present inventor has isolated and identified a unique HA binding protein which is a component of a novel HARC. It has been shown that this binding protein or HA receptor is involved in cell locomotion or motility. A novel cDNA encoding the HA receptor has been molecularly cloned in the present invention.

35 The inventor has also identified a novel amino acid sequence motif which comprises the HA binding regions of the locomotion-associated HA receptor and is also common to other extracellular and cell-associated HA binding proteins.

In accordance with one aspect of the invention, an isolated DNA molecule is provided comprising a nucleotide sequence encoding a locomotion-associated hyaluronan binding protein.

5 In accordance with a further aspect of the invention, an isolated protein is provided comprising a locomotion-associated hyaluronan binding protein.

10 In accordance with a further aspect of the invention, a hyaluronan-binding amino acid sequence motif is provided having a formula:



wherein X^1 and X^2 are the same or different and are basic amino acid residues and wherein A_n is an amino acid sequence comprising seven or eight amino acid residues 15 which are the same or different and are neutral or basic amino acid residues.

In accordance with further aspects of the invention are provided peptides having the formulae:

20 Lys - Gln - Lys - Ile - Lys - His - Val - Val - Lys;

Lys - Ile - Lys - His - Val - Val - Lys - Leu - Lys;

25 Lys - Leu - Arg - Ser - Gln - Leu - Val - Lys - Arg; and

Lys - Leu - Arg - Ser - Gln - Leu - Val - Lys - Arg - Lys.

30

DETAILED DESCRIPTION OF THE INVENTION

The integrity of complex organisms depends not only upon their resistance to foreign pathogenic organisms but on tight regulation of the differentiation, growth and 35 locomotion of cells comprising tissues. For instance, aberrant regulation of locomotion contributes to host response to tissue injury, expression of birth defects

and neoplasia. The molecular mechanisms that regulate cell locomotion are only now being investigated. In the past, attention focused upon the role of adhesive molecules and proteins of the contractile cytoskeleton in 5 motility. Although these components are essential for locomotion, we are now understanding that they are not sufficient.

It is becoming clear that cell locomotion must be initiated and is likely not restrained by physical 10 barriers such as cell-cell contact and basement membranes as was previously supposed. An increasing number of molecules are being described that are potential candidates for the initiation of locomotion. These include autocrine motility factors, growth factors and 15 components of the extracellular matrix. One component of the extracellular matrix that has particularly been implicated in cell locomotion is the glycosaminoglycan hyaluronic acid or hyaluronan. The production of this polysaccharide has been correlated to the migration of 20 embryonic, tumour and lung fibroblasts in a number of laboratories.

Oncogenic transformation by both activated src and ras genes promotes synthesis of HA (29,34) and the growth of many human tumours is accompanied by elevated levels 25 of this glycosaminoglycan in the serum or in tissue surrounding the tumour (25,27). Tumour cells often show increased responsiveness to HA-stimulating factors (4,13) and, recently, the increase in locomotion of tumour cells, particularly ras-transformed cells, has been shown 30 to be mediated by HA (34).

Several investigators have demonstrated that hyaluronan interacts with the cell surface via high affinity specific receptors. A number of receptor candidates have been described but only two cell surface 35 hyaluronan receptors have been molecularly characterized, CD44 (23) and the novel HA receptor to be described. These proteins appear to be unrelated to one another.

Utilizing antibodies specific to the 52, 58 kDa proteins of HARC, the inventor has isolated and characterized a novel 2.9Kb cDNA from a λGT11 3T3 cell cDNA expression library that encodes a 48 or 52 kDa protein, depending upon the initiation codon used. This protein is unique, occurs on the cell surface, is regulated by the mutant H-ras oncogene and mediates locomotion of ras-transformed cells responding to HA.

10 Antibodies to peptide sequences encoded by the cDNA block the increase in locomotion resulting from induction of the mutant H-ras gene. In a transblot assay, the bacterially expressed protein binds to biotinylated HA. Its unique structure and HA binding properties indicate that it is a new HA receptor. In view of its role in cell locomotion, the protein is referred to as RHAMM, an acronym for Receptor for HA Mediated Motility. RHAMM binds to HA with high affinity ($K_D = 10^{-8}M$) and specificity.

15 RHAMM is down-regulated in stationary normal cells and is only expressed in situations where cell motility is required, for example, in wound healing, in response to growth factors and in chemotaxis by white blood cells.

20 Using monoclonal antibodies specific for RHAMM, it has been demonstrated that it is a tightly regulated protein that is expressed just prior to and immediately following initiation of cell motility in vitro. Furthermore, antibodies to this protein both prevent

25 initiation of locomotion and inhibit rapidly locomoting cells (34,53).

Increased expression of RHAMM has also been observed in several types of tumour cells, including breast tumours, lymphomas and leukemia (96,97).

30 RHAMM and the HARC complex of which it is a component have been found to be present in all cells

examined, in situations where locomotion is involved, including chicken fibroblasts (28) and frog neurites.

These results suggest that the interaction of HA and RHAMM is a universal mechanism for regulating cell 5 locomotion.

As indicated above, RHAMM is a unique protein and its discovery is novel since it has not previously been described at the molecular level. Study of its properties has led to the novel proposal that cell 10 locomotion is initiated and can therefore be molecularly predicted. Further, stimulation appears to occur via messenger mechanisms and not necessarily via alterations of cell adhesion.

The detailed study of the HA binding regions of 15 RHAMM, and the effect of deletions and point mutations on HA binding have enabled the inventor to identify a novel amino acid sequence motif which is not only responsible for HA binding by RHAMM but is present in all other HA binding proteins examined.

20 The novel amino acid sequence motif associated with HA binding comprises a sequence of 9 or 10 amino acids in which the first and last amino acids are basic amino acids, either lysine or arginine, and the intervening seven or eight amino acids are neutral or basic amino acids. If the two terminal basic amino acids flank an intervening sequence of less than seven or more than 25 eight amino acids, HA binding is lost.

The primary amino acid sequence of the intervening seven or eight amino acids is unimportant, provided no 30 acidic amino acids are included. Inclusion of an acidic amino acid in the intervening sequence either abolishes HA binding or reduces it substantially.

The characterisation of RHAMM and of its constituent HA binding domains and the cloning and sequencing of its 35 cDNA provide tools for controlling or regulating the interaction of HA and RHAMM and therefore for controlling

or regulating HA-stimulated locomotion in vertebrate cells, including mammalian cells.

Antibodies to RHAMM or to one or more of its HA binding domains block HA binding and inhibit cell locomotion. Since RHAMM/HA interaction is involved in oncogene-and growth factor mediated-cell locomotion, antibodies to RHAMM, or to variants or fragments thereof which retain HA binding ability, provide means for therapeutic intervention in diseases involving cell locomotion, for example, tumour invasion, birth defects, acute and chronic inflammatory disorders, Alzheimer's and other forms of dementia, including Parkinson's and Huntington's diseases, AIDS, diabetes, auto immune diseases, corneal displasias and hypertrophies, burns, surgical incisions and adhesions, strokes, multiple sclerosis, depression/schizophrenia related to neuronal growth and pain states involving nerve sprouting.

Other situations involving cell locomotion, in which intervention using antibodies to RHAMM or its constituent peptides could be employed, include CNS and spinal cord regeneration, contraception and in vitro fertilisation and embryo development. The inventor has found that antibodies to RHAMM inhibit human sperm motility in vitro and also inhibit fertilisation of hamster ova by human sperm in an in vitro system. Antibodies to RHAMM or to fragments thereof or to its constituent binding peptides may be made by conventional methods known to those skilled in the art.

HA stimulated locomotion may also be controlled by regulation of expression of RHAMM, for example, by use of antisense DNA sequences directed to the RHAMM gene or portions thereof or by gene therapy, for example by homologous recombination using RHAMM cDNA.

RHAMM HA-binding peptides, such as peptide^{aa 404-411} and peptide^{aa 423-432} may also be employed to interfere with and control RHAMM/HA interaction. These peptides probably act by removing the ligand, HA.

12.

It has also been demonstrated that RHAMM binds to the enzyme responsible for HA synthesis, HA synthase, which is located on the cell surface, and by binding reduces HA production (93). HA-stimulated cell 5 locomotion may therefore be controlled by administration of solubilised RHAMM protein to control HA production.

In view of the tight regulation of RHAMM in normal cells, it provides a useful diagnostic marker for cancer, inflammatory and autoimmune diseases, trauma and recovery 10 from tissue injury associated with vascular disease, toxins or drugs and for predicting birth defects from diagnosable mutations in the RHAMM gene or gene regulating expression of RHAMM.

Insertion of the correct gene would permit 15 correction of diagnosed birth defects associated with abnormalities of the RHAMM gene.

The protein encoded by the DNA of the invention appears to be identical or related to the 58 and 52 kDa proteins of a hyaluronan receptor complex previously 20 reported (29). These proteins have been shown to be involved in locomotion (2,34) and, recently, to also bind to HA (35). Thus, a polyclonal antibody specific to the encoded protein crossreacts with the 52, 58 kDa HARC protein. Conversely, a battery of monoclonal antibodies 25 that are specific for the 52 and 58 kDa HARC proteins crossreact with bacterially expressed RHAMM. These observations combined with the ability of the HARC proteins to reverse the blocking effect of Pab specific to the encoded protein on cell locomotion (Fig. 5), the 30 precise co-localization of the two antigens, their common regulation by the H-ras oncogene, their ability to bind to HA and to mediate ras-regulated locomotion indicate that the gene product isolated corresponds to a component of HARC.

35 Although the cDNA encoding the 58 kDa protein does not contain a strong signal sequence, the inventor has shown by ultrastructural localisation studies that the

protein is located at the cell surface. Although somewhat unusual, a similar lack of signal sequences has been noted for other cell surface receptors including lymphocyte Fc receptor for IgE (10), transferrin receptor (15), liver asialoglyco-protein receptor (9), and the high affinity laminin receptor (18,36).

Blocking Pab and Mab antibodies to HARC were prepared and used to screen a λ gt11 3T3 cDNA expression library (Clonetech, Palo Alto, CA) as described in Example 1. A restriction map was constructed using the enzymes (U.S. Biochemical Corporation, Cleveland, OH) indicated in the restriction map of Figure 1. The open reading frame of the clones is boxed. The sequencing strategy is shown below the cDNA clones. Both antibodies to peptides I and II encoded in the cDNA (peptide I : nucleotides 372-435; peptide II : nucleotides 804-864), and a radiolabeled ACC I fragment of the 1.9 kb insert were used to isolate the 2.9 complete cDNA. The amino acid sequences are shown above the DNA sequences in Figure 1. Two possible initiation codons are indicated with highlighted letters. The cDNA encodes a 48 or 52 kDa protein, depending on which initiation codon is used. Potential N-glycosylation sites are marked with asterisks and possible signal sequences are underlined with broken lines. The stop codon is indicated by highlighted letters.

Restriction mapping and sequencing of the 2.9 Kb insert demonstrated that it encodes a complete protein and contains the original 1.9 Kb cDNA sequence in its central region (Figure 1). The sequence is unique and does not bear significant homology to other proteins registered in NERB or EMBL data banks or to factors known to be involved in ras regulated locomotion (14). Like previously characterized proteins such as p53 (37), it contains two possible initiation codons, encoding proteins of either 52.2 kDa or 46.7 kDa respectively (Fig. 1B). The encoded protein is rich in glutamic acid,

lysine, glutamine and leucine. It has a Pi of 5.2, is hydrophilic and most of the polypeptide is predicted to occur as an alpha helix by Chou Fasman analyses (5). The most notable feature of the deduced sequence is a 21 5 amino acid stretch (which corresponds to Peptide I,) that is repeated 5 times near the N-terminus. The predicted protein contains 8 putative N-glycosylation sites, 5 of which are concentrated within the repeated motif. The protein also contains clusters of positively charged 10 amino acids throughout the open reading frame. It does not encode a hydrophobic sequence long enough to span the plasma membrane and possible signal sequences following either initiation codon are weak (Fig. 1).

15 The Encoded Protein Occurs at the Cell Surface

In spite of an absence of a consensus, hydrophobic region encoded in the cDNA, the protein occurs at the cell surface as demonstrated by ultrastructural localization of immunogold to the cell membrane (Fig. 2), 20 and by live cell immunofluorescence using Pab to Peptide II. At the light microscope level, the encoded protein is seen to strikingly accumulate in the ruffles and processes of H-ras transformed cells (Fig. 3). It also occurs intracellularly in the perinuclear region (data 25 not shown). This distribution is typical of molecules that regulate cell locomotion (1,14). It is further noted that Pab I and II co-localize precisely with Mab to the 58 kDa HARC protein.

Zinc sulphate induction of a metallothionein- 30 regulated mutant H ras gene transferred into 10T $\frac{1}{2}$ fibroblasts (26) gave increased expression of a 58 kDa protein detected by Pab to Peptide II, as seen in Figure 4A, as well as increased expression of p21 Ras proteins (Figure 4B). These results indicate that RHAMM 35 expression is regulated by the mutant H-ras gene. The induction of the mutant ras gene gave increased

expression of a 5.2 kb mRNA transcript, as seen in Figure 4C.

5

Antibodies to Peptides Encoded in the RHAMM cDNA Block Locomotion

Direct evidence for a role of the encoded protein in H-ras regulated cell locomotion was demonstrated in 10 experiments designed to test whether antibodies to the encoded protein inhibited cell locomotion. As noted previously (34), induction of the mutant H-ras gene with zinc sulfate activated an HA-dependent motility mechanism in mutant H-ras transfected fibroblasts (Fig. 5). 15 Antibodies to peptide II specifically inhibited ras regulated locomotion (Fig. 5). As well, transfection of a RHAMM genomic clone into 10T½ fibroblasts resulted in overexpression of RHAMM at the cell surface and an 8-fold increase in the rate of cell locomotion (data not shown). 20

Bacterially Expressed RHAMM binds to biotinylated HA

A 1.3 Kb insert encoding RHAMM (insert containing the open reading frame from the second initiation codon) was expressed in E. coli, as described in Example 1.

25 RHAMM was identified on immunoblots, using Mab 3T3-5, specific for the 52,58 kDa HARC proteins, as a 70 kDa protein, as seen in Figure 6, Lane D. The expressed protein specifically bound to biotinylated HA (Lane A) and the binding was competed with by excess unlabelled HA 30 (Lane B). Bacterial lysates from bacteria containing plasmids without the insert did not show HA binding (Lane C).

In transblot immunoassays utilising purified HARC proteins as substrate, it was shown that Pab to peptide I 35 cross-reacted with the 58 and 52 kDa proteins of HARC (Figure 7, Panel C). The locomotion blocking antibody Mab 3T3-5 also cross-reacted with these proteins (Figure

7, Panel B). These results show the antigenic relationship between the protein encoded by the cDNA of the invention and the 52,58 kDa HARC proteins, which are the HA binding proteins of the complex (35).

5 A complete RHAMM cDNA (1.43 kb) was expressed as a fusion protein with pGEX-2T in E. coli HB101 and was shown to bind specifically to biotin-labelled HA and to antibodies specific for RHAMM in a transblot assay, as seen in Figure 8, and to HA-Sepharose. The complete cDNA
10 was truncated with restriction endonucleases from the 3' end resulting in 1.30 kb, 1.02 kb, 0.71 kb and 0.41 kb cDNAs which were then expressed in HB101. The truncation strategy is shown in Figure 9. Only the fusion peptide expressed from the complete cDNA and the 1.30 kb cDNA
15 bound to HA, as seen in Lanes 6 and 5, Figure 10B, indicating that the region located between 1.02 kb to 1.30 kb of RHAMM cDNA is critical for recognition of this glycosaminoglycan. Deletion of 114 bases in this region virtually eliminated HA binding activity thus defining
20 the major glycosaminoglycan binding region to amino acids 400-434 located near the carboxy terminus of RHAMM, as seen in Figures 12A and B. Two domains containing clusters of basic amino acids were identified within this region, peptide ^{aa}401-411 and peptide ^{aa}423-432. Synthetic
25 peptides corresponding to these two domains both inhibited HA binding to the complete 1.43 kb expressed GST-RHAMM fusion protein, and also directly bound to HA-Sepharose, as seen in Figure 13A and B. Random peptides and peptides mimicking other regions of RHAMM did not
30 inhibit HA:RHAMM interactions and bound weakly to HA-Sepharose. Oligonucleotides encoding either of these two peptides were linked to the N-terminal 0.71 kb of RHAMM which encoded a peptide that did not contain HA binding activity. Fusion proteins containing either of these
35 recombinant peptides acquired HA binding activity as assessed with a transblot assay, shown in Figure 14B.

The inventor has shown by study of the HA binding domains of RHAMM that the structural features required for HA binding comprise a novel amino acid sequence motif, a sequence of nine or ten amino acids, with a 5 basic amino acid in each terminal position and the intervening positions being either basic or neutral amino acids, but not acidic amino acids.

Three such motifs occur in RHAMM, peptide^{aa401-410}, peptide^{aa402-411} and peptide^{aa423-431}.

10 The presence of further basic amino acids, in addition to the terminal positions, either within the motif, or adjacent to the motif, will give increased binding affinity to HA.

15 The inventor's studies indicated that the positions of the basic amino acids in the HA binding domains of RHAMM are important.

20 Since HA is composed of repeating disaccharide units of N-acetyl glucosamine and D-glucuronic acid, it was predicted that each tetra- to hexa- saccharide unit of HA was the functional unit for HA to bind to receptors 25 spanning at least two carboxyl groups of HA. Each HA binding domain in previous results had approximately 10 amino acid residues and both of them had α -helical folding, fitting with this model (91). The distance of each disaccharide unit (or distance of two negative charges) spaces approximately 9 amino acids. Thus, the presence of basic amino acids at 1 and 9 or 10 positions will increase the binding affinity to HA since the interaction is ion dependent. Both HA binding domains of 30 RHAMM have basic amino acids at 1 and 9 positions (two basic amino acids spaced apart by 7 amino acids). The random peptides shown to have much lower affinity to HA-Sepharose had no basic amino acids at positions 1 and 9.

35 RHAMM has, however, five domains in which two basic amino acids are separated by seven amino acid residues. These are peptide^{aa54-62}, peptide^{aa90-98}, peptide^{aa98-106}, peptide^{aa227-235}, and peptide^{aa320-328} (53). These domains do not have the

ability to bind HA since the deletion of the two HA binding domains, peptide^{aa401-411} and peptide^{aa423-432}, abolished the HA binding activity of the remaining polypeptide (Fig. 12B, lane 3). It was noted that acidic amino acids 5 were present between the two basic amino acids in all of these five domains that did not bind to HA. This suggested that acidic amino acids were not compatible with HA binding, since charges are the basis of the interaction.

10 The inventor has shown that deletion of peptide^{aa423-432} along with partial deletion of peptide^{aa401-411} from RHAMM (Figure 22) completely destroyed the ability of the receptor to bind to HA, indicating that other regions of the protein do not bind to HA, even though other domains 15 have two basic amino acids separated by seven intervening amino acid residues.

20 To further test the inventor's HA-binding motif model, a number of synthetic peptides were prepared and their HA-binding ability was determined using HA-sepharose. A tyrosine residue was added to one end of each synthetic peptide for convenient spectrophotometric quantitation.

25 The results are shown in Figure 16. Peptide B (Y K Q K I K H V V L L L) had low ability to bind to HA-sepharose; this peptide has a basic amino acid at position 1 but none at position 9 or 10. Peptide A (Y K Q K I K H D D K L K) also had low ability to bind to HA-sepharose; this peptide has acidic amino acids between the basic amino acids at positions 1 and 9. Peptide D (Y 30 R V R G V L G K R R) had the highest ability to bind to HA-Sepharose; this peptide contains both requirements for HA binding, basic amino acids at positions 1 and 9 and no intervening acid amino acids. The ability of peptide C (Y K Q K I K H V V L K L) to bind to HA-Sepharose was 35 quite high, probably due to the presence of basic amino acids at positions 1 and 10.

The importance of the key basic amino acids for HA binding to RHAMM was pinpointed by point mutation of these amino acids, with accompanying loss of HA binding.

Figure 17A shows the strategy of site-directed 5 mutations of peptide¹²⁴⁻²³⁻⁴³² employed. Panel B lists the resulting mutated amino acid sequences.

DNA inserts coding for peptide¹²⁴⁻²³⁻⁴³² or for a mutation were inserted into pGEX-2T and transformed into HB101. Fusion proteins expressed from these DNA inserts were 10 examined for HA binding, as shown in Figure 18.

The results show that the HA-binding ability of the first 3 site-directed mutated samples was crippled (Figure 18C, lane 2-4, mutations I-III), and that of the last site-directed mutated sample was completely 15 destroyed (lane 5, mutation IV).

The results indicate that change of neutral amino acid V to an acidic amino acid D (Fig. 18C, lane 3) slightly decreased its HA-binding ability. The change of a basic amino acid to a neutral amino acid decreased HA-binding ability seriously (Fig. 18C, lanes 2 and 4). The change of KRK to HHH completely destroyed HA-binding ability (Fig. 18C, lane 5). These results demonstrate that basic amino acids are crucial in HA-binding motifs. Acidic amino acids negatively affect HA binding ability. 25 His is apparently not important in HA binding; substitution of His for Arg or Lys abolishes HA binding.

Studies of HA binding proteins other than RHAMM have indicated that sequences containing clusters of basic amino acids are involved in HA binding, eg. tandem repeat 30 sequence in cartilage proteoglycan (83,84) and rat link protein (48,7).

Examination of the published amino acid sequences of various HA-binding proteins, however, disclosed no homology of amino acid sequence which could be linked to 35 HA binding.

The inventor has recognised the novel amino acid sequence motif described above as the binding domains of

RHAMM and has also identified this unique motif in all reported sequences of HA binding proteins.

These findings are set out in Table 1. In Section II of Table 1 are listed the binding regions of various 5 HA-binding proteins as reported in the literature, along with the primary amino acid sequence of the novel HA-binding sequence motif of the invention identified within these binding regions.

10 As shown in Section II of Table 1, link protein (8), versican (92), hyaluronidase (HAase, 98) and aggrecan (7) all contain one or more HA-binding motifs, with either lysine or arginine in positions 1 and 9.

15 The inventor has identified the unique HA binding motif at peptide²⁷¹⁻²⁷⁹ of the G1 domain of rat cartilage proteoglycan core protein, the G1 domain having been shown to be an HA binding domain by Doege et al. (7). The G2 domain of rat cartilage proteoglycan core protein has been shown not to bind HA (7), although containing 20 similar amounts of basic amino acids (9% in G1 and 8% in G2). The recognition by the inventors of the HA binding motif explains the lack of HA binding of the G2 domain as the critical motif does not occur in G2.

25 The inventor has performed genetic manipulations on link protein and on CD44 and confirmed the HA-binding ability of the putative binding domains identified by recognition of the novel sequence motif of the invention. These binding sequences are shown in Table 1, Section I.

Link protein contains an HA-binding motif at 30 peptide²³¹⁶⁻³²⁵ and at peptide²³¹⁰³⁻¹¹². Attachment of link protein peptide²³¹⁶⁻³²⁵ to a part of the RHAMM polypeptide that does not bind HA, as described in Example 3, conferred HA-binding properties on that peptide.

35 The results are shown in Figure 19B.

A tandem repeat loop of link protein has been demonstrated to be an HA binding domain by solid-phase assay using immobilised HA and synthetic peptides (8).

The inventors have identified the unique HA binding motif in the tandem repeat loop of link protein, at peptide^{aa 316-325} and confirmed by genetic manipulation that this sequence is an HA binding domain (Figure 21).

5 Genetic manipulation of CD44 was also carried out. A domain R R R C G Q K K K (peptide^{aa 292-300}) of CD44 was aligned by PCR technique to the 5' region of RHAMM cDNA (nucleotide 1-375) that encodes a non-HA-binding polypeptide. The strategy is shown in Figure 20. This
10 CD44 domain was then mutated to R R R C G Q K K Q using primer for mutation 1 and to R R R C G Q E E Q using primer for Mutation 2. Fusion proteins were prepared from selected clones and subjected to Western blot assays stained with anti-RHAMM antibody or biotinylated HA (data
15 not shown).

When peptide^{aa 292-300} was linked to the N-terminal domain of RHAMM, the fusion protein bound HA. When amino acid 294 or 295 was mutated, the HA-binding ability of the fusion protein was destroyed.

20

EXAMPLES

EXAMPLE 1

25 The following description contains details of methods used and results obtained by the inventor in the isolation and characterization of RHAMM.

The following abbreviations are used:

30 GAG, glycosaminoglycan; Pab, polyclonal antibody; Mab, monoclonal antibody; SDS, sodium dodecyl sulfate; HRP, horse radish peroxidase; TBS, tris buffer saline; TRIS, Trizma HCL.

Antibodies

35 Polyclonal (Pab) and monoclonal (Mab) antibodies to HARC were prepared as described previously (28,29). All antibodies were purified by affinity chromatography on HARC-Sepharose and/or Protein A-Sepharose. Mab 3T3-5, which blocks fibroblast locomotion, was originally used

to screen the cDNA library. Mab 3T3-5 is shown here to recognize 52-58 kDa proteins in SDS-immunoblots of lysates of ras transformed fibroblasts and has previously been shown to block HA-promoted locomotion of these 5 fibroblasts (29). Mab 3T3-7, which was used for immunofluorescence, reacts specifically with the 52-58 kDa proteins of HARC, as shown here by SDS immunoblot analyses, but does not block HA-promoted locomotion (29). Synthetic peptides mimicking two amino acid sequences in 10 the deduced sequence [Peptide I : nucleotide sequence 372-435 of Figure 1 (corresponding to a portion of the repeated sequence) and Peptide II : nucleotide sequence 804-864 of Figure 1] were produced by the Peptide Analysis Core of the University of Alabama Cancer Center 15 (Birmingham). The peptides were purified by HPLC and characterized by amino acid composition analyses. Antibodies to the peptides were raised in rabbits as described (28,29). These antibodies were purified by affinity chromatography on columns of the appropriate 20 peptides coupled to CNBr-activated Sepharose and the specificity of purified anti-peptide antibodies to the respective peptide was confirmed by dot blot assays. Preimmune antibodies were obtained from each rabbit or mouse prior to immunization, purified as described above 25 and used as controls.

Cloning and DNA sequencing

A 3T3 cDNA library in λ gt11 was obtained from 30 Clontech (Palo Alto, CA) and initially screened with both Pab and Mab (3T3-3) to HARC. One clone, which gave a positive signal upon repeated screening with the antibodies, had a 1.9 kb insert that was subcloned into pUC18 and M13.

Sequencing of the insert revealed an open reading 35 frame corresponding to a 340 amino acid residues of a C-terminal protein segment that did not include an initiation codon (Fig. 1). Additional clones coding for

the same protein were isolated by rescreening the library with a radiolabelled Acc 1 restriction fragment of the 1.9 cDNA and with Pab to peptide sequences (peptide I and II, Fig. 1) encoded in the insert. A 2.9 Kb clone was 5 positive in both screenings and was further characterized (Figure 1).

The restriction map was constructed using the enzymes indicated in Figure 1 (U.S. Biochemical Corporation, Cleveland, OH). Nucleotide sequences were 10 determined by the dideoxy chain termination method using Sequenase I and Sequenase II kits (U.S. Biochemical Corporation, Cleveland, OH). Digestion of the insert with Acc 1 yielded a fragment which was radiolabelled with a Random priming kit (BRL, Bethesda, MD) and was 15 used to rescreen the cDNA library at high stringency. The inserts were isolated after digestion of the λphage with Eco R1 and ligated into pUC18 and M13 for sequencing. Sequence analysis and comparisons were conducted using IBI/Puskell sequence analysis software 20 and NBRF database (version 18, 4). Additional homology searches were conducted with NBRF (6/90) and EMBL (5/90) using FASTA and word search programs in GCG software.

Ultrastructural Localization of Encoded Protein

25 For ultrastructural localization of the encoded protein, H-ras expressing tumor cell monolayers were processed with A) Pab to Peptide II (0.5 µg/ml) or (B) preimmune IgG, (0.5 µg/ml) followed by gold conjugated goat anti-rabbit IgG (5 nm, Sigma Chemicals, St. Louis, 30 MO). Antibody localized gold particles to the cell surface (magnification 30,780 fold). Sections incubated with pre-immune sera did not bind particles (magnification 30,780 fold) (Fig. 5).

Immunofluorescent Localization of Encoded Protein Relative to HARC Proteins

Double immunofluorescence studies of zinc-induced fibroblasts were performed as described (28,32) using (a) Mab 3T3-5 which specifically reacted with 52-58 kDa proteins of HARC and (b) Pab to Peptide II (nucleotide sequence 804-864). The Mab was detected with FITC-anti-mouse IgG and the Pab was detected with RITC-anti-rabbit IgG.

Secondary antibodies were rhodamine labelled goat anti-mouse IgG (to detect Mab 3T3-7) and fluorescein labelled goat anti-rabbit IgG (to detect Pab to Pep. 1) and were purchased from Sigma Chemicals (St. Louis, MO). Processed monolayers were examined with a Zeiss IM35 equipped with epifluorescence utilizing non-overlapping filters of 510-560 nm (for rhodamine) and 450-490 nm (for fluorescence). Lack of bleedthrough was confirmed by examination of single immunofluorescence samples with both filters (data not shown).

Both antibodies were strikingly localized in the ruffles and processes of zinc-sulfate induced fibroblasts as seen in Figure 3. Pre-immune sera showed no immunofluorescence (data not shown). Magnification 6000 fold.

Immunocytochemistry and Electron Microscopy

Fibroblasts transfected with the mutant ras gene were grown overnight on glass coverslips that had been treated with Teflon and baked at 250°C for 30 min. Cells were incubated with primary antibodies (0.5 µg/ml, Pab to Pep I) or preimmune IgG (0-5 µg/ml) for 1 hr and then 25 incubated with gold-conjugated goat anti-rabbit IgG (5 nm, Sigma Chemicals, St. Louis, MO), fixed with 0.1% glutaraldehyde in PBS and postfixed in 4% osmium tetroxide (31,33). Cultures were dehydrated through alcohol series, infiltrated with Spurr resin (1:1 resin:alcohol for 2 h at 37°C) and embedded in 100% Spurr for 12 h at 60°C. The glass coverslips were removed from the hardened resin and ultra-thin sections were obtained

with a diamond knife (IVIC, 2.0 mm). Sections were examined with a Phillips 300 transmission electron microscope.

5 Expression of encoded protein is regulated by the mutant H-ras oncogene.

Cell lysates were prepared from buffer (-) and zinc sulfate treated (+) 10T $\frac{1}{2}$ fibroblasts transfected with mutant H-ras under the control of a metallothionein 10 promoter (26) as described previously (34). Proteins were fractionated on SDS-PAGE (12.5%) transferred to nitrocellulose and probed with the primary antibodies, Pab to Peptide II (nucleotide sequence 804-864, 0.1 μ g IgG/ml) or RAS-10 antibody, the results being shown in 15 Figure 4, Panels A and B respectively.

Isolation of RNA and Northern Assays

Confluent cultures of 212 cells were exposed to zinc sulphate or to buffer alone (34) for 24 hours. Total RNA 20 was extracted according to Choy et al. (6). 60 μ g of RNA was electrophoresed on 1% agarose gels and transblotted onto Nytran Nylon membranes. The mRNA transcript for the encoded protein was detected using 32 P-labelled 1.2 kb fragment of the cDNA that contained the open reading 25 frame, the results being shown in Figure 4C. Blots were reprobed with 32 P-GAPDH to control for RNA loading and the results were shown in Figure 4D.

Locomotion Assays

30 A 10T $\frac{1}{2}$ cell line containing an inducible EJ-ras metallothionein-neomycin hybrid gene (26) was used for quantitating the effect of both Pab to peptide II and Mab to HARC on cell locomotion in response to HA as previously described (34). Antibodies (Mab 3T3.5, 1 μ g/ml, Pab Peptide II, 1 μ g/ml) were added to zinc 35 induced (24 hr), HA treated cells, $\frac{1}{2}$ hr before filming, then cells were filmed by video timelapse for a total of

2 hrs. To assess specific effects of the antibodies, they were preincubated for 1 hr at 4°C with 50 µg of HARC proteins prior to their addition to control locomotion assays. Addition of these proteins by themselves had no 5 effect on cell locomotion (results not shown). Data were analyzed with a Zeiss Dynacell program that utilized Fourier analysis of cell locomotion to derive a cell motility index (17).

The effect of non-immune sera, Mab 3T3-5 to HARC (1 10 µg/ml), and Pab to Peptide II (nucleotide sequence 804-964, 1 µg/ml) on HA-promoted locomotion was analyzed. Both Mab 3T3-5 to HARC and Pab to Peptide II inhibited cell locomotion in response to HA. This effect was reversed by the addition of excess HARC proteins. 15 Non-immune sera had no effect on locomotion relative to controls. Values represent the mean + S.E.M. N=50 cells. The results are shown in Figure 5.

Expression of RHAMM in Bacteria

20 Oligoprimers corresponding to the second methionine were prepared and the open reading frame from this start codon was amplified with PCR. The second initiation codon was chosen since the size of the protein generated by N-glycanase digestion of the 58 kDa protein (data not shown) closely matched that predicted using the second 25 initiation codon (35). PCR generated a 1.3 Kb DNA fragment that was cloned into the PGEX-2 T vector (22). The vector was used to transform JM101 E.Coli and expression was induced by adding 0.2mM isopropyl-beta-D-thiogalactopyranoside (IPTG) as described (22). Isolated 30 bacterial cells were sonicated in a 0.5 mM Tris (pH 8.0) buffer containing 4M urea, 1% triton x-100 and 1mM EDTA. The urea was removed by a two step dialysis procedure. The sonicate was initially dialized for 6 hours at 4°C 35 into phosphate buffered saline (pH 7.4) containing 1% triton. The soluble material was applied to a glutathione-agarose affinity gel as described (22) and

bound protein was eluted with 0.2 mM glutathione in 0.05 M, Tris (pH 8.0). The purified, expressed protein was then electrophoresed on 12.5% SDS-PAGE.

5 Hyaluronan Binding Assay

Lysates from bacteria either with plasmids containing an insert encoding RHAMM or with plasmids without the insert were prepared by sonication. Twenty-five micrograms of lysate protein were 10 electrophoresed on 10% SDS-PAGE and transblotted onto nitrocellulose membranes. Additional binding sites were blocked with 5% defatted milk and incubated for 1 hour with or without biotinylated HA prepared as described (35). The membrane was washed for 1 hour in Tris-buffered saline containing 0.05% TWEEN then incubated 15 with streptavidin-HRP (1:1000 dilution Sigma Chemicals). The bound streptavidin was visualized with chemiluminescence (Amersham ELL) according to instructions.

20

Bacterially expressed RHAMM binds to biotinylated HA

Bacterial plasmids containing a 1.3 Kb insert encoding RHAMM (Figure 6, Panels A,B,D) or no insert (Figure 6, Panel C) were sonicated, electrophoresed on 25 SDS-PAGE, transblotted onto nitrocellulose and the remaining protein binding sites blocked with casein as described above. Biotinylated HA was added to the blot in the absence (A) or presence of 100-fold excess unlabelled HA (B). Bound HA was then detected with 30 streptavidin-HRP and visualized with chemiluminescence. Bacterial lysates containing the 1.3 Kb insert were incubated with monoclonal antibody 3T3-5 (D) to the 58, 52 kDa HARC proteins and visualized as described. These results (Figure 4) show that HA bound to the expressed 35 protein and binding was competitive with unlabelled HA. MAB 3T3-5, that blocks locomotion, specifically recognized the expressed protein. The molecular weight

standards are marked with arrow heads and include $\alpha 2$ macroglobulin (180 kDa); B-galactosidase (116 kDa); Fructose-6-phosphate kinase (84 kDa); pyruvate kinase (58 kDa) and fumarase (48.5 kDa).

5

SDS-Immunoblots

Immunoblot assays were conducted on isolated soluble HARC proteins (29) or cell lysates prepared from H-ras transfected fibroblasts exposed to either zinc sulphate 10 or buffer alone for 24 hours. The latter were prepared with lysis buffer containing 25 mM Tris, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA and protease inhibitors (29). Proteins were fractionated by SDS-PAGE on 12.5% polyacrylamide gels 15 (29) and transferred to nitrocellulose membranes (Biorad, Rockville Center, NY). Additional protein binding sites were blocked with 0.5% defatted milk in Tris buffered saline (TBS, 34) and the membrane was incubated with primary antibodies (1 μ g IgG/ml of TBS containing 0.5% 20 defatted milk) for 12 hr at 4°C on a gyratory shaker (Stovall, Greensboro, NC). The monoclonal antibody to p21 ras (pan, ras-10) was purchased from Dupont. Primary antibodies were detected by chemiluminescence as described in instructions (ECL, Amersham). Briefly, the 25 washed membranes were incubated with horseradish peroxidase (HRP) labelled IgG secondary goat antibody for 20 min to 1 hr at room temperature. After washing, the membranes were developed with peracid followed by luminol and an enhancer. The oxiacid product was detected by 30 immediately exposing with Kodac X-Omat Xac-5 film.

Immunoreactivity of anti-peptide antibody to the encoded Protein

HARC proteins were purified from spent culture media 35 as described (29) then fractionated by SDS-PAGE and transblotted onto nitrocellulose filters. HARC proteins were visualized with India ink (Figure 7, Panel A) and

have MWe of 72, 68, 58 and 52 kDa (arrows). The 52-58 kDa proteins reacted specifically with B) Mab 3T3-5 (blocker of locomotion) to HARC (Panel B), and Pab to Peptide I, nucleotide sequence 372-435, (Panel C).
5 Molecular weight protein standards are marked by arrowheads and include from the top of the gel, phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and trypsin inhibitor (21.5 kDa).

10 Example 2

Construction of RHAMM cDNA

The RHAMM cDNA reading frame was amplified with Polymerase Chain Reaction (PCR) (61) using two oligonucleotides as primers: one complimentary to the 15 translation initiation region (nucleotide 1-22) creating a BamHI site linked to nucleotide 1; the other complementary to the region 280 bases after the translation stop codon (nucleotide 1685-1706) creating an EcoRI site linked to nucleotide 1706. The full length 20 cDNA inserted in Bluescript (Stratagene, La Jolla, CA) was used as a template. The reaction was carried out at 94°C (30 sec), 54°C (30 sec), and 72°C (90 sec), for a total of 25 cycles (61). The PCR product (1.7 Kb) was digested with proteinase K (50 µg/ml) in 0.5% SDS at 37°C 25 for 30 min. DNA was extracted from the mixture with an equal volume of phenol:chloroform (1:1), an equal volume of chloroform, then ethanol precipitated (61). The PCR product was doubly digested with EcoRI and BamHI and purified by agarose gel electrophoresis. The EcoRI-BamHI 30 DNA fragment was ligated to an EcoRI and BamHI opened pGEX-2T plasmid (62,22, Pharmacia).

The above ligation mixture was transformed into competent E. coli HB101 cells as described (61,63). Colonies were streaked on a master LB/amp agar plate and 35 a replica was prepared by overlaying a Nylon Hybond N1' membrane on the LB/amp agar plate. Both plates were incubated at 37°C for 3 h. The master plate was kept at

4°C. The Hybond N' membrane was screened with RHAMM cDNA as a probe (53). The hybridization was carried out at 65°C in a solution containing 6 x SSC, 5 x Denhardt's solution, 0.5% SDS, and 20 µg denatured salmon sperm DNA per ml hybridization solution overnight as described (22). The membrane was washed at 65°C with 2 x SSC, 1 x SSC, 0.1 x SSC (30 min for each washing) and exposed to Kodak X-Omat film as described (22). Positive colonies, which indicated the presence of a RHAMM cDNA insert, were recovered from the master plate, and the presence of a correct RHAMM cDNA insert was confirmed with appropriate restriction endonuclease digestion and agarose gel electrophoresis to determine the size of the insert.

15 Fusion Protein Preparation

Colonies of pGEX-2T plasmids containing RHAMM cDNA were grown in 5 ml LB/amp medium at 37°C overnight. 0.1 mM isopropylthio- β -D-galactoside (IPTG) (100 mM stock solution in water) was added to the cultures (0.5 mg protein/ml) to induce the biosynthesis of fusion proteins (63). The cultures were grown for 90 min in the presence of this inducer. The cells were harvested by centrifugation and lysed in 1 ml 50 mM Tris-Cl, pH 8.0, containing 2 M Urea and 1% Triton X-100 (53). Cells were further disrupted by sonication and centrifuged at 15,000 xg for 20 min to separate soluble from insoluble fractions. The supernatants that contained the GST-RHAMM fusion protein were recovered and used for Western blot assay.

30

Identification of Fusion Protein

GST-RHAMM fusion protein was prepared from lysates of colonies of HB101 containing the RHAMM cDNA and electrophoresed on SDS-PAGE as an 83 kDa protein (Fig. 8). On Western blot assay, this fusion protein reacted with i) polyclonal antibodies to a peptide encoded in RHAMM cDNA (peptide¹²⁵⁻¹⁴⁵) (53) (Fig. 8, lane 2), ii) a monoclonal

antibody specific to RHAMM (Fig. 8, lane 4) and iii) biotin-labelled HA (Fig. 8, lane 6). Bacterial lysates containing GST but not the RHAMM cDNA plasmid did not react with these reagents (Fig. 8, lanes 1,3,5). These results 5 confirmed the identity of the fusion protein as RHAMM and showed that it bound to biotin-labelled HA as previously reported (53). The ability of RHAMM fusion protein to bind to HA was also confirmed by its ability to bind to HA-Sepharose (data not shown).

10

Truncation of RHAMM cDNA

The RHAMM cDNA was cloned into the pGEX-2T plasmid (62,22) as described above. This plasmid contains cloning sites that are followed by three stop codons at three 15 different reading frames (62,22). The recombinant plasmid was digested with restriction endonucleases (a) AatII, (b) BglII + EcoRI, (c) Ncol + EcoRI and (d) SacI + EcoRI. Digests were blunt ended with the Klenow Fragment or, for the EcoRI-SacI fragment, S1 nuclease (Fig. 9). Plasmids 20 containing each truncated RHAMM cDNA respectively were recovered by agarose gel electrophoresis, self-ligated and then transformed into HB101 cells as above. Positive clones were selected by hybridization with RHAMM cDNA and confirmed to contain truncated RHAMM cDNAs by restriction 25 endonuclease digestion and agarose gel electrophoresis to quantitate insert size.

An additional deletion of RHAMM cDNA was achieved using the restriction endonucleases EcoRI and HphI. Plasmid pGEX-2T containing the complete RHAMM cDNA was 30 digested with EcoRI and then blunt ended with the Klenow Fragment. DNA was recovered by ethanol precipitation, suspended in TE buffer, pH 8.0, digested with BglII and purified by agarose gel electrophoresis. The blunt ended EcoRI-BglII fragment (5.66 kb), was purified with a Prep-A-35 Gene Kit. Conversely, the complete RHAMM cDNA, obtained from the recombinant plasmid, was digested with HphI, blunt ended with S1 nuclease and precipitated with ethanol. DNA

was resuspended in TE buffer, pH 8.0, digested with BglII and purified by agarose gel electrophoresis. The blunt-ended HphI-BglII fragment (477 bp) was isolated, purified with a Prep-A-Gene Kit, ligated to the blunt-ended EcoRI-BglII fragment and transformed into HB101. Eight colonies that hybridized with RHAMM cDNA were picked to prepare DNA. DNA samples were digested with BglII and Tth3I to confirm that the correct insert (0.41kb, 0.71kb, 1.02kb and 1.3kb) was present.

10 The truncated RHAMM cDNAs used in these experiments are shown in Figure 9.

15 The truncated RHAMM cDNA plasmids were transformed into HB101 and expressed as fusion proteins of 43, 55, 67 and 83 kDa and were detected using anti-GST antibodies (Fig. 10A). These proteins corresponded to the size of protein predicted from the truncations shown in Fig. 9.

20 Truncated fusion proteins were tested for their ability to bind to biotin-labelled HA (Fig. 10B). The complete fusion protein bound HA (lane 6) as did the SacI truncation (lane 5), but the fusion proteins expressed from the NcoI (lane 2), BglII (lane 3) and AatII (lane 4) truncated RHAMM cDNAs did not stain with biotin-labelled HA. These results indicated that the NcoI-SacI fragment containing a 95 amino acid sequence near the carboxy 25 terminus was critical for binding to HA.

Effect of salt concentration

30 It has previously been postulated that positive charge clusters are important in binding glycosaminoglycans, particularly heparin (854-60). To test whether or not charge is important in the binding of HA to RHAMM, the effect of increasing salt concentration on binding of biotin-labelled HA to expressed RHAMM fusion protein was determined.

35 GSTRHAMM fusion protein was separated on SDS-PAGE and transblotted onto nitrocellulose membrane. Proteins were stained with biotin-labelled HA in the presence of

increasing NaCl (150-2000 mM). Results are shown in Figure 11.

As has been determined for heparin-protein interactions (58), the binding of the fusion protein product to biotin-labelled HA was reduced to 19% of the control binding activity when the NaCl concentration was increased from 150 mM to 2,000 mM. These results indicated a role for ionic interactions in HA:RHAMM binding.

10 Immunoblots and Transblots using Biotinylated HA

The method described originally by Towbin (63) was used for preparing immunoblots with the following minor modifications. Proteins were separated on 10% SDS-PAGE and transblotted onto nitrocellulose membranes in a buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3. Additional protein binding sites on the membrane were blocked in 10 mM Tris-Cl, pH 8.0, containing 150 mM NaCl (TBS), 0.05% Tween 20 (TBST) and 5% skim milk powder (TBSTS) for 1 h. The blocked membrane was incubated with primary antibody diluted in TBSTS for 1 h at room temperature or overnight at 4°C. The membrane was washed with TBST for 4 x 10 min, and then incubated with the appropriate secondary antibody conjugate diluted in TBST. After washing as above, the membrane was rinsed with TBS. Antibody binding was visualized with a chemiluminescence method (ECL) (64,65) used according to the manufacturer's, instructions (Amersham).

The labelling of hyaluronan with biotin was a modification of the procedure for labelling of antibodies with biotin (66). This method incorporates label onto free amino groups which are present in small amounts within the hyaluronan molecule (67). HA, at an average MW_E 500,000 daltonis, was dissolved in 0.1 M sodium borate, pH 8.8, (1.5 µg/µl), incubated with N-hydroxysuccinimide biotin dissolved in DMSO (7.5 µg/µl) at a ratio of 50:1 (HA:Biotin v/v) at room temperature

for 4 h and then incubated with 15 μ l of 1 M ammonium chloride for 10 min. The solution was dialysized against PBS, pH 7.2 and then PBS containing 30% glycerol. The linkage of biotin to HA was stable for several months at 5 4°C and preparation and properties of this compound are described in detail elsewhere (68). It is believed to link to HA via the few amino groups present in this glycosaminoglycan (67). Staining of cell lysates with biotinylated HA was carried out as described above except 10 that labelled HA was substituted for primary antibody and diluted in TBST.

Identification of two HA binding motifs

Two sequences encoded in the NcoI-SacI fragment (nucleotide ¹²⁰¹⁻¹²³³ and nucleotide ¹²⁶⁷⁻¹²⁹⁶) that was shown to bind to HA in the above section were noted to contain clusters of positive charges. To determine whether these motifs were involved in the binding of HA to RHAMM, three approaches were used. In the first approach, the region 15 was deleted and then the resulting expressed truncated fusion protein was tested for its ability to bind to HA (Fig. 12A&B). In the second approach, peptides were synthesized that mimicked the two regions containing positive charge clusters and then tested for their 20 ability to HA-Sepharose and to compete with RHAMM for binding biotin-labelled HA in a transblot assay (Fig. 13A&B). In the third approach, oligonucleotides encoding 25 the putative binding domains were genetically engineered, using PCR, to the 0.71 Kb cDNA (non-HA binding) encoding the amino terminus of RHAMM. The altered fusion protein 30 products were then tested for their ability to bind to HA (Fig. 14A&B).

In the first approach, a deletion of HphI-SacI cDNA fragment that contained the clusters of basic amino acids 35 was made as described above. A correct religation of the deleted cDNA was determined by restriction mapping. The DNA sample having a BglII site and a shorter fragment

after BglIII-Tth3I digestion implied that a deletion of HphI-EcoRI fragment had occurred and that a correct religation ensued (Fig. 12A). The colonies containing this correct religated plasmid were used to make GST-fusion protein as described above. The GST-RHAMM fusion protein was electrophoresed as a 74 kDa protein, which was the size predicted for this deletion. In a transblot procedure using biotin-labelled HA, the 74 kDa deleted protein had lost its ability to bind to HA (Fig. 12B, lane 3). This result defined the critical HA binding site of RHAMM as the region between aa 400-434 occurring within the HphI and SacI sites marked in Fig. 9.

The fusion protein from RHAMM cDNA truncated with SacI and EcoRI showed binding to HA (Figure 12B, lane 2).

To further assess the role of the deleted region in binding to HA, peptides corresponding to the two domains containing clusters of basic amino acid residues, $K^{401}Q\ K\ I$ $K\ H\ V\ V\ K\ L\ K^{411}$, $K^{423}L\ R\ S\ Q\ L\ V\ K\ R\ K^{432}$, were synthesized and used to compete with binding of biotinylated HA to the complete GST-RHAMM fusion protein.

Transblotted RHAMM fusion protein was stained with a 1:10,000 dilution of biotin-labelled HA that had been pre-incubated for 1 h with 3 mg/ml BSA (Figure 13A, lane 1), 3 mg/ml peptide^{aa401-411} (lane 2) or 3 mg/ml peptide^{aa423-432} (lane 3). Results showed that peptide^{aa401-411} and peptide^{aa423-432} blocked the binding of biotin-labelled HA to expressed GST-RHAMM fusion protein.

To directly demonstrate the ability of peptide^{aa401-411} and peptide^{aa423-432} to bind to HA, peptides were chromatographed on HA-Sepharose.

HA-Sepharose affinity gel was prepared according to the manufacturer's instruction. RHAMM peptides (peptide^{aa401-411}; peptide^{aa423-432}; randomized peptide^{aa401-411} (L K Q K V K K H I V); randomized peptide^{aa423-432} (Q S K R L K K R V L); peptide^{aa125-145} and peptide^{aa269-288}, (53) were applied to HA-Sepharose. Unbound peptides were removed by washing the gel with PBS containing 0.15 M NaCl. The amounts of

peptides applied and the unbound peptide removed from the gel were determined by measuring their O.D. value. Results are shown in Figure 13B.

Several controls were included to evaluate the role 5 of non-specific binding. BSA, as well as a peptide^{aa125-145} mimicking a repeated sequence encoded in the RHAMM amino terminus and a peptide^{aa269-288} mimicking a hydrophilic region encoded in the center of the RHAMM cDNA (53) were all tested for their ability to bind to HA-Sepharose. As 10 well, to investigate the importance of the spacing of basic amino acids, two random peptides that contained the same amino acid residues as peptide^{aa401-411} and peptide^{aa423-432} were also tested for their ability to bind to HA sepharose. These 15 results showed that only peptide^{aa401-411} and peptide^{aa423-432} efficiently bound to HA (Fig. 13B).

DNA sequences encoding peptide^{aa401-411} and peptide^{aa423-432} were each linked to the BglII truncated RHAMM cDNA which encodes the 27.5 kDa polypeptide of the N-terminus of 20 RHAMM.

Construction of Recombinant RHAMM Containing Oligonucleotides Encoding HA-Binding Peptides

PCR was used to incorporate the HA binding regions 25 (peptide^{aa401-411} and peptide^{aa423-432} respectively) into a cDNA encoding the N-terminus of RHAMM that was prepared as a 0.71 Kb fragment (aa 1-238, see above and Fig 9). The fusion protein product of this fragment did not have the ability to bind HA (Fig. 14). The procedure was carried 30 out by making two PCR primers (5'TAG AAT GAA TTC TTT CAA TTT CAC AAC ATG TTT GAT TTT TTG TTT AAG ATC TTC TAT TTC and 5'TAG AAT GAA TTC TTT CCT TTT AAC AAG CTG AGA TCG CAG TTT AAG ATC TTC TAT TTC) which contained both a region mimicking the oligonucleotides encoding either peptide^{aa401-411} 35 and peptide^{aa423-432} (creating an EcoRI site at the end of each primer) and a region mimicking 18 bp of the 3' end cDNA of the 0.71 Kb insert. Recombinant cDNA was

obtained with a PCR reaction by using either of these two primers together with a primer that mimicked the 5' end of the RHAMM cDNA (nucleotide 1-22) (creating a BamHI site) with the same conditions described in the 5 construction of RHAMM cDNA. Both PCR products were digested with EcoRI and BamHI and purified in 1% agarose gel electrophoresis. Recombinant cDNAs were then inserted into pGEX-2T opened with BamHI and EcoRI and transformed into HB101 as above. The correct insertion 10 of the recombinant cDNAs was confirmed by restriction endonuclease digestion of the selected clones and by sizing of the insert with agarose gel electrophoresis.

These recombinant sequences were then expressed as recombinant proteins (Fig 14A). The HA binding ability 15 of the resulting recombinant proteins was tested in transblot assays.

Bacterial cell lysates containing the GST-RHAMM fusion proteins were fractionated on SDS-PAGE, transblotted onto nitrocellulose membrane and visualized 20 with either polyclonal antibody to peptide^{aa125-145} (lane 1-3) or biotinlabelled HA (lane 4-6). The GST fusion non-recombinant polypeptide^{aa1-238} was used as a control (lane 1 and lane 4). The linkage of either peptide^{aa401-411} (lane 2 and lane 5) or peptide^{aa423-432} (lane 3 and lane 6) to the N-terminal RHAMM polypeptide^{aa1-238} created HA binding domains 25 (lane 5-6) although their antibody binding properties remained the same (lane 2-3).

Truncated RHAMM fusion proteins containing either the peptide^{aa401-411} (lane 5) or the peptide^{aa423-432} (lane 6) 30 bound efficiently to HA (Fig 14B). In contrast, and as noted in Fig 10B, the fusion protein containing only the amino terminus of RHAMM did not bind to HA (Fig. 14B, lane 4). These results indicated that each genetically engineered alteration of the amino terminus encoded in 35 RHAMM cDNA created an HA binding site, indicating that both peptide^{aa401-411} and peptide^{aa423-432} comprise the HA binding domains of RHAMM.

Blocking of HA Binding with RHAMM Peptides

GST-RHAMM fusion protein was electrophoresed on 10% SDS-PAGE and transblotted onto a nitrocellulose membrane. The membrane was cut into 3 strips and each was stained

5 with 1:3000 diluted biotin-labelled HA in the presence of 3 mg/ml BSA, 3 mg/ml peptide²⁴⁰¹⁻⁴¹¹ and 3 mg/ml peptide²⁴²³⁻⁴³², respectively. The other steps were the same as described in immunoblots.

10 Inhibition of HA-stimulated locomotion by RHAMM peptides

Peptides K⁴⁰¹ Q K I K H V V K L K⁴¹¹ and K⁴²³ L R S Q L V K R L⁴³² were synthesised and their effect on HA-stimulated locomotion in ras-transformed fibroblast cells was examined.

15 Peptides (1 ng to 500 µg/ml) were added to cells and their effect on cell locomotion was monitored with an imager analysis system as described (53).

The results are shown in Figure 15.

20 Hyaluronan-Sepharose Affinity Chromatography

Hyaluronan (Sigma) was coupled to AH-Sepharose 4B (Pharmacia) according to the manufacturer's instructions, resulting in an HA-Sepharose gel. Peptides, ordered from Coast Scientific (San Diego) were HPLC purified. These and randomized peptides, whose sequence was generated by computer, were applied to HA-Sepharose in the presence of 0.15 M NaCl in PBS buffer. Unbound peptides were removed by extensive washing with the same buffer. The amounts of peptides applied and that removed were determined by measuring optical density (O.D.) at 214 nm.

EXAMPLE 3

Materials

Biotin-labelled HA was prepared by dissolving the 35 polymer (1.5 µg/µl) in 0.1 M sodium borate, pH 8.8, incubating with N-Hydroxysuccinimide biotin dissolved in DMSO (7.5 µg/µl) at a ratio of 50:1 (HA:Biotin) at room

temperature for 4 h and then incubating the reacted products with 15 μ l of 1 M ammonium chloride for 10 min. The solution was dialysed against PBS, pH 7.2 and then PBS containing 20% glycerol. The biotin-labelled HA 5 could be stored at 4°C for several months. Staining of cell lysates with biotinylated HA was carried out as described below (transblot assay) except that labelled HA was substituted for primary antibody and diluted in TBST.

Peptides were synthesized by Dr. David Litchfield, 10 Manitoba Institute of Cell Biology. The purity of synthetic peptides were determined on HPLC at optical densities (OD) 280 and 214. Oligonucleotides were synthesized in the Dept. of Physiology, University of Manitoba. Oligonucleotides longer than 30 bases were 15 purified on HPLC. AH-Sepharose power was obtained from Pharmacia.

Polymerase Chain Reaction (PCR)

PCR reactions were performed as previously described 20 (61) using a standard (P)[?] kit (Amersham). The reaction mixture (100 μ l) normally contained 1.25 nM MgCl₂, 75 μ M dNTP, 1 μ g of each primers (in excess), 5 ng template DNA and 1 unit of Taq DNA polymerase. All reactions were carried out at 94°C (30 sec), 54°C (30 25 sec) and 72°C (90 sec) for 25 cycles.

DNA Purification and Ligation

DNA products from the PCR reaction mixtures were purified using Prep-A-Gene DNA Purification kit. The 30 reaction mixture was brought to 1 ml by adding 25 μ l Prep-A-Gene Matrix and 875 μ l binding buffer. After washing twice in binding buffer and 3 times in wash buffer, the DNA was eluted in 100 μ l H₂O. The DNA was 35 ligated to plasmids as described (94) using a DNA ligase (Boehringer Mannheim).

Transformation, DNA Preparation and Digestion with
Restriction Endonucleases

Competent bacteria were prepared as described (61) with some modifications. Bacteria (*E. coli* HB101 or XL1-5 Blue), stored in glycerol, were grown in 1 ml LB/amp medium at 37°C overnight. The resultant culture was brought to 10 ml with LB/amp medium and Mg₂SO₄ was added to a concentration of 10 mM. The culture was incubated at 37°C for 2 h with constant agitation. The cells were 10 chilled on ice for 20 min. pelleted at 1,000 xg for 5 min at 4°C, resuspended in 3 ml 50 mM CaCl₂, re-chilled on ice for 20 min, pelleted at 1,000 xg for 5 min at 4°C, resuspended in 500 µl 50 mM CaCl₂, and put on ice for transformation. Transformation was carried out exactly 15 as previously described (61). The transformed mixtures were spread on 3 LB/amp agar plates for HB101 or 3 LB/amp agar plates containing isopropylthio-β-D-galactoside (4 mg/plate) and x-gal (0.2 mM/plate) for XL1-Blue. The plates were incubated at 37°C overnight. Twelve white 20 colonies of XL1-Blue or twelve randomly picked colonies of HB101 were streaked on LB/amp agar plate for incubation at 37°C overnight and inoculated in Eppendorf tubes containing 1 ml LB/amp medium for incubation at 37°C overnight with shaking at 280 rpm.

25 DNA's were prepared from the above 1 ml culture as described (61) with modifications. The cells were sedimented and resuspended in 100 µl solution I. Cells from each tube were lysated with µg lysozyme dissolved in 10 µl solution I and with 200 µl 0.2 N NaOH containing 30 1% SDS at room temperature for 10 min. 150 µl Solution III was added to each lysate and put on ice for 15 min. Genomic DNA and bacterial debris were sedimented with 10,000 xg for 15 min. Plasmid DNA in the supernatant was precipitated with 0.6 volume of isopropyl alcohol at room 35 temperature for 10 min and then by centrifugation at 10,000 xg for 15 min. The DNA was resuspended in 30 µl TE buffer, pH 8.0, and purified with Prep-A-Gene kit

using 5 μ l Prep-A-Gene Matrix washed twice with binding buffer and twice with wash buffer. The purified DNA was eluted in 50 μ l H₂O.

DNA samples were doubly digested with two restriction endonucleases (i.e. BamHI+Sacl, BamHI+EcoRI, BamHI+ClaI or ClaI+EcoRI) and electrophoresed on an agarose gel to select those clones containing correct inserts. For experiments using Bluescript, the correct insert was recovered for further use by Prep-A-Gene DNA Purification kit. For experiments using pGEX-2T (62,22), the clones containing the correct insert were used for preparation of fusion proteins.

Site-directed mutagenesis of HA binding domain
15 (peptide^{aa423-432}) in RHAMM.

Four cDNAs containing site-directed mutations in each cDNA were generated in four PCR reactions. The template DNA was the part of RHAMM cDNA (nucleotide 1-714) containing the oligonucleotide encoding peptide^{aa423-432} of RHAMM. The primers in each reaction were the one complimentary to translation initiation region and one of the 4 primers containing mutated nucleotides as in Figure 17. PCR products from the 4 primers containing mutated nucleotides were doubly digested with BamHI + EcoRI, ligated into pGEX-2T and transformed into HB101. Selected clones were confirmed to contain the inserts by double digestion with BamHI + EcoRI and electrophoresis on agarose gel, as shown in Figure 18A. Fusion proteins were prepared from the selected clones, proceeded to Western blot and stained with anti-RHAMM antibody, (Figure 18B) and with biotin-labelled HA (Figure 18C).

30 Genetic manipulation of link protein peptide^{aa316-325}

Peptide^{aa316-325} of link protein (R Y P I S R P R K R) has been shown to contain an HA binding domain (8).

An oligonucleotide encoding peptide^{aa316-325} of link protein was aligned to a part of RHAMM cDNA encoding a polypeptide that did not bind to HA, polypeptide^{aa1-238}, using PCR techniques, as shown in Figure 19A.

5 This was carried out by having a primer that was complimentary to nucleotide 358-375 of RHAMM and nucleotide 946-975 of link protein creating an EcoRI site. Both of them were in the same reading frame. The recombinant cDNA was generated using this primer and 10 another primer complementary to nucleotide 1-22 of RHAMM creating a BamHI site at nucleotide 1. The PCR product was then doubly digested with BamHI + EcoRI, ligated into pGEX-2T, transformed into HB101 and confirmed to be present in the selected clones. The strategy is 15 illustrated in Figure 19A. Western Blot assays of the fusion proteins obtained from these clones were performed and the results are shown in Figure 19B.

Fusion proteins were prepared from the original clone containing RHAMM peptide^{aa1-238} only (lane 1 and 4) and 20 the clone additionally containing peptide^{aa316-325} of link protein (lanes 2 and 5). HB101 lysate was used as a control (lanes 3 and 6). The fusion proteins on Western blot assay were stained with anti-RHAMM antibody (lanes 1-3) and biotin-labelled HA (lanes 4-6). The results 25 showed that only fusion protein containing peptide^{aa316-325} of link protein had the ability to bind to biotin-labelled HA (lane 5).

Site-Directed mutagenesis of HA binding domain (peptide^{aa401-411}) in RHAMM

The strategy of the site-directed mutagenesis is shown in Figure 17.

Two primers within peptide^{aa401-411} were synthesized which generated 3 site mutations as required and created 35 a ClaI site in each primer (Figure 18). Combined with the other two primers which were used to amplify the complete RHAMM cDNA reading frame, the RHAMM cDNA were

generated into two pieces overlapped with a ClaI site in two PCR reactions. The two ClaI sites were in the same reading frame of the cDNA. After PCR amplification, Part I (Figure. 18A, lane 1) was doubly digested with BamHI + 5 ClaI and ligated into BamHI-ClaI opened Bluescript. Part II (Fig. 18A, lane 2) was doubly digested with ClaI + EcoRI and ligated into ClaI-EcoRI opened Bluescript. The ligation mixtures were transformed into E. coli XL1-Blue. DNAs were prepared from white colonies and doubly 10 digested by BamHI + ClaI or ClaI + EcoRI to select the clones containing appropriate inserts (Fig. 18A, lane 3 and 4). The inserts were recovered with Prep-A-Gene kit, doubly ligated into BamHI-EcoRI opened Bluescript and proceeded as above to select a proper insert containing 15 Part I and Part II (Fig. 18A, lane 5).

The insert was recovered and ligated into BamHI-EcoRI opened pGEX-2T and transformed into HB101. The clone containing expected insert identified by restriction endonuclease digestion (Fig. 18A, lane 6) was 20 selected and used to prepare fusion protein. Fusion proteins (Fig. 18B, lanes 1 and 3) were subjected to Western blot assay stained with antibody to RHAMM (Fig. 18B, lanes 1-2) and biotin-labelled HA (Fig. 18B, lanes 3-4). Non-mutated RHAMM fusion protein was used as 25 control (Fig. 18B, lanes 2 and 4). The results (Fig. 18B, lane 4) showed that after the alteration of Lys⁴⁰⁵, Lys⁴¹¹ and Lys⁴²³ to Glu⁴⁰⁵, Glu⁴¹¹ and Glu⁴²³, the HA binding ability of RHAMM was severely compromised.

30 Site-directed mutation of RHAMM

21A. Part I and Part II were amplified in PCR reactions (lane 2 and 3).

21B. The PCR products were doubly digested with BamHI + ClaI (for Part I) and ClaI + EcoRI (for Part II), 35 ligated to properly opened Bluescript and transformed into E. coli XL1-Blue. DNAs were prepared from white colonies and confirmed to contain appropriate inserts by

digestion with proper restriction endonucleases (lane 2 and 3). The two DNAs were doubly ligated into a Bluescript and confirmed to be present in the selected clones (lane 4). This insert, containing the mutated 5 complete RHAMM cDNA was recovered and ligated into pGEX-2T and confirmed to present in that plasmid by digestion with BamHI + EcoRI (lane 5).

10 Peptide^{aa401-411} and peptide^{aa423-432} are the only HA binding domains in RHAMM

These two peptide sequences of RHAMM are close together and followed by an unique SacI site within the RHAMM cDNA reading frame (Fig. 1). RHAMM cDNA from nucleotide 1-1230 was amplified in PCR. In this way, HA 15 binding domain peptide^{aa423-432} was completely deleted while the other HA binding domain peptide^{aa401-411} was partially deleted. The basic amino acids in the remaining domain were mutated (Fig. 1) to completely abolish its HA binding ability. The PCR product was inserted into pGEX- 20 2T containing SalI-EcoRI fragment and transformed into E. coli HB101. DNA samples prepared from randomly picked clones were confirmed to contain correct inserts.

The colony containing a correct BamHI-SacI insert 25 was used to made fusion proteins. Undeleted RHAMM fusion protein and HB101 lysate were used as controls. The fusion proteins were subjected to Western blot assays stained with antibody to RHAMM and biotin-labelled HA (data not shown). The results showed that the deleted fusion protein completely lost its ability to bind to HA 30 indicating that these two HA binding domains were the only HA binding domains in RHAMM.

The present invention is not limited to the features 35 of the embodiments described herein, but includes all variations and modifications within the scope of the claims.

Table 1

HA-BINDING MOTIFS

<u>PROTEINS</u>	<u>BINDING REGION</u> (amino acids)	<u>SEQUENCE</u>	<u>REF.</u>	<u>SEQUENCE</u> <u>I.D. NO.</u>
RHAMM	401-411	KQKIKHVVKLK	3	
RHAMM	423-432	KLKSQLVKRK	4	
LINK PROTEIN	316-325	RYPISRPRKRR	22	
CD44	292-300	RRRCGQKKKK	23	
I. Identified by Molecular Manipulation				
LINK PROTEIN	103-112	RKSYGKYQGR	8	
VERSICAN	2319-2327	KTFGKMKPR	92	
AGGRECAN	71-79	RIKWSRVSK	7	
AGGRECAN	2109-2117	KRTMRPTRR	7	
HAsE	96-104	RGTRSGSTR		
HAsE	106-117	RRRKKIQGRSKR	98	
II. Predicted Domains				

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I claim:

1. An isolated DNA molecule comprising a nucleotide sequence encoding a locomotion-associated hyaluronan binding protein.
2. A DNA molecule in accordance with claim 1 encoding the derived amino acid sequence of Figure 1 commencing with the first methionine, or a variant or fragment thereof which retains the ability to bind hyaluronan.
3. A DNA molecule in accordance with claim 1 encoding the derived amino acid sequence of Figure 1 commencing with the second methionine, or a variant or fragment thereof which retains the ability to bind hyaluronan.
4. A DNA molecule in accordance with any of claims 1 to 3 wherein the DNA molecule is a cDNA molecule.
5. A DNA molecule having the nucleotide sequence of Figure 1.
- 25 6. A DNA molecule having the nucleotide sequence of nucleotides 147-1428 of Figure 1.
7. An isolated protein comprising a locomotion-associated hyaluronan binding protein.
- 30 8. A protein in accordance with claim 7 having the derived amino acid sequence of Figure 1 commencing with the first methionine, or a variant or fragment thereof which retains the ability to bind hyaluronan.
- 35 9. A protein in accordance with claim 7 having the amino acid sequence of Figure 1 commencing with the

second methionine, or a variant or fragment thereof which retains the ability to bind hyaluronan.

10. A protein in accordance with any of claims 8 or 5 wherein the protein is a recombinant protein.

11. A hyaluronan-binding amino acid sequence motif having a formula:

$$X^1 - A_n - X^2$$

10 wherein X^1 and X^2 are the same or different and are basic amino acid residues and wherein A_n is an amino acid sequence comprising seven or eight amino acid residues which are the same or different and are neutral or basic amino acid residues.

15

12. A sequence motif in accordance with claim 11 wherein X^1 and X^2 are the same or different and are selected from the group consisting of lysine and arginine and wherein n is seven.

20

13. A DNA molecule comprising a DNA molecule in accordance with any of claims 1 to 6 inserted into a replicable cloning vehicle.

25

14. A DNA molecule in accordance with claim 13 wherein the cloning vehicle is a bacterial plasmid.

15. A peptide having the formula:

30 Lys - Gln - Lys - Ile - Lys - His - Val - Val - Lys.

16. A peptide having the formula:

Lys - Ile - Lys - His - Val - Val - Lys - Leu - Lys.

17. A peptide having the formula:

Lys - Leu - Arg - Ser - Gln - Leu - Val - Lys - Arg.

5 18. A peptide having the formula:

Lys - Leu - Arg - Ser - Gln - Leu - Val - Lys - Arg - Lys.

10 19. A peptide in accordance with any of claims 15
to 18 wherein the peptide is a synthetic peptide.

20. An isolated DNA molecule encoding a sequence
motif in accordance with any of claims 11 or 12.

15 21. An isolated DNA molecule encoding a peptide in
accordance with any of claims 15 to 19.

22. An antibody specific for a protein in
accordance with any of claims 7 to 10.

20 23. An antibody specific for a peptide in
accordance with any of claims 15 to 19.

25 24. An antibody specific for an amino acid sequence
motif in accordance with any of claims 11 or 12.

30 25. A pharmaceutical composition comprising an
effective amount of at least one antibody specific for a
protein in accordance with any of claims 7 to 10 and a
pharmaceutically acceptable carrier.

35 26. A pharmaceutical composition comprising an
effective amount of at least one antibody specific for a
peptide in accordance with any of claims 15 to 19 and a
pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising an effective amount of at least one antibody specific for an amino acid sequence motif in accordance with any of claims 11 or 12 and a pharmaceutically acceptable carrier.

28. A pharmaceutical composition comprising an effective amount of a protein in accordance with any of claims 7 to 10 or a variant or fragment thereof and a pharmaceutically acceptable carrier.

29. A pharmaceutical composition comprising an effective amount of a peptide in accordance with any of claims 15 to 19 and a pharmaceutically acceptable carrier.

30. A method of controlling locomotion of cells in a vertebrate comprising administering to the vertebrate an effective amount of at least one antibody in accordance with claim 22 to 24.

31. A method in accordance with claim 30 wherein the cells are inflammatory cells, keratinocytes, tumour cells or fibroblasts.

32. A method of controlling locomotion of vertebrate cells during in vitro fertilisation and embryo development comprising administering to the cells an effective amount of at least one antibody in accordance with any of claims 22 to 24.

33. A method of treating a disease associated with altered cell locomotion in a vertebrate comprising administering to the vertebrate an effective amount of at least one antibody in accordance with any of claims 22 to 24.

34. A method of controlling binding of hyaluronan to vertebrate cells comprising contacting the cells with an effective amount of at least one antibody in accordance with any of claims 22 or 24.

5

35. A method of controlling locomotion of cells in a vertebrate comprising administering to the vertebrate an effective amount of a peptide in accordance with any of claims 15 to 19.

10

36. A method of treating a disease associated with altered cell locomotion in a vertebrate comprising administering to the vertebrate an effective amount of a peptide in accordance with any of claims 22 to 24.

15

37. A method of inhibiting fertilisation of ova by sperm in a vertebrate comprising contacting the sperm with at least one antibody specific for RHAMM or for a variant or fragment thereof.

20

38. A method of controlling locomotion of cells in a vertebrate comprising administering to the vertebrate an effective amount of a protein in accordance with any of claims 7 to 10 or a variant or fragment thereof.

25

39. A method of treating a disease associated with altered cell locomotion in a vertebrate comprising administering to the vertebrate an effective amount of a protein in accordance with any of claims 7 to 10 or a variant or fragment thereof.

30

40. A method of treating a disease associated with altered cell locomotion in a vertebrate comprising administering to the vertebrate an effective amount of a DNA molecule in accordance with any of claims 1 to 6.

60

41. A method of controlling locomotion of cells in a vertebrate comprising administering to the vertebrate an effective amount of RHAMM so as to control synthesis of hyaluronan.

5

42. A method in accordance with any claims 30 to 41 wherein the vertebrate is a mammal.

43. A method in accordance with any claims 30 to 41
10 wherein the vertebrate is a human.

44. A method of detecting a disease or
physiological state associated with altered expression of
RHAMM in the cells of a vertebrate comprising obtaining a
15 sample of the cells and determining the presence or
amount of RHAMM or a fragment thereof in the cells.

1/23

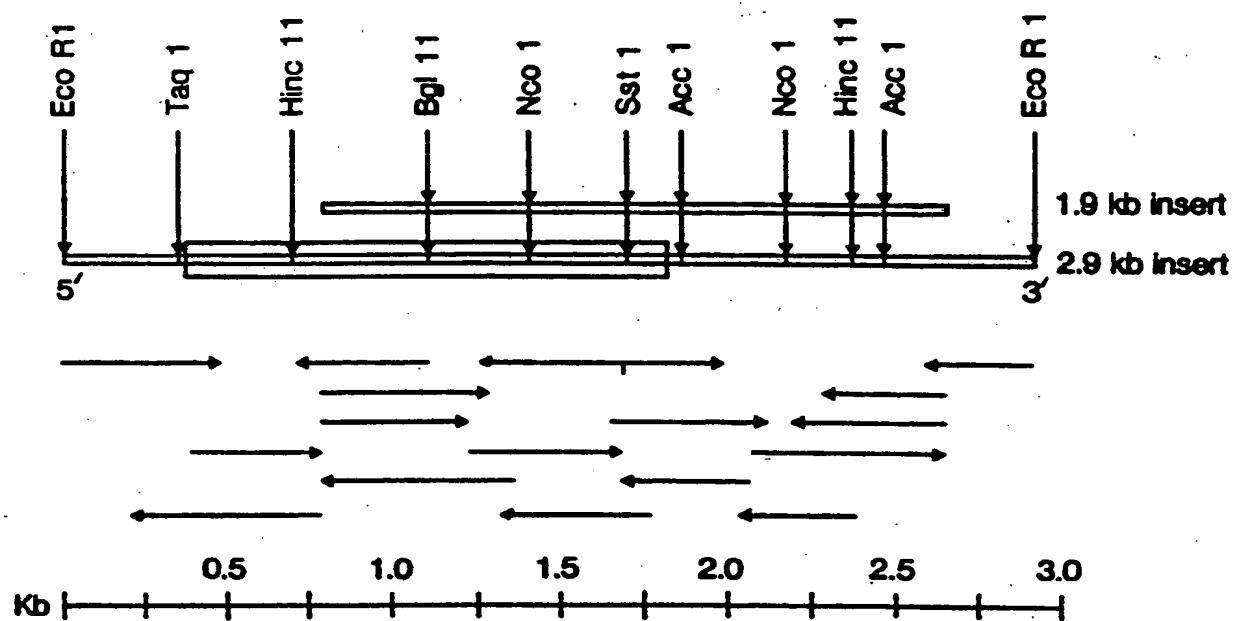


FIGURE 1

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2 / 23



FIG.2a.

FIG.2b.



FIG.3a.



FIG.3b.

3/23

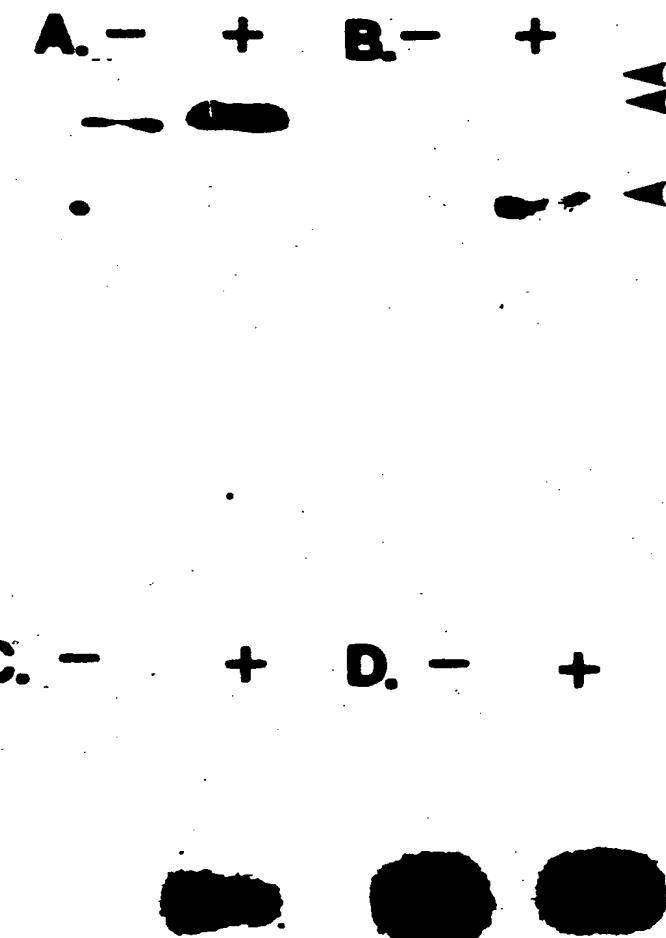


FIG.4.

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4/23

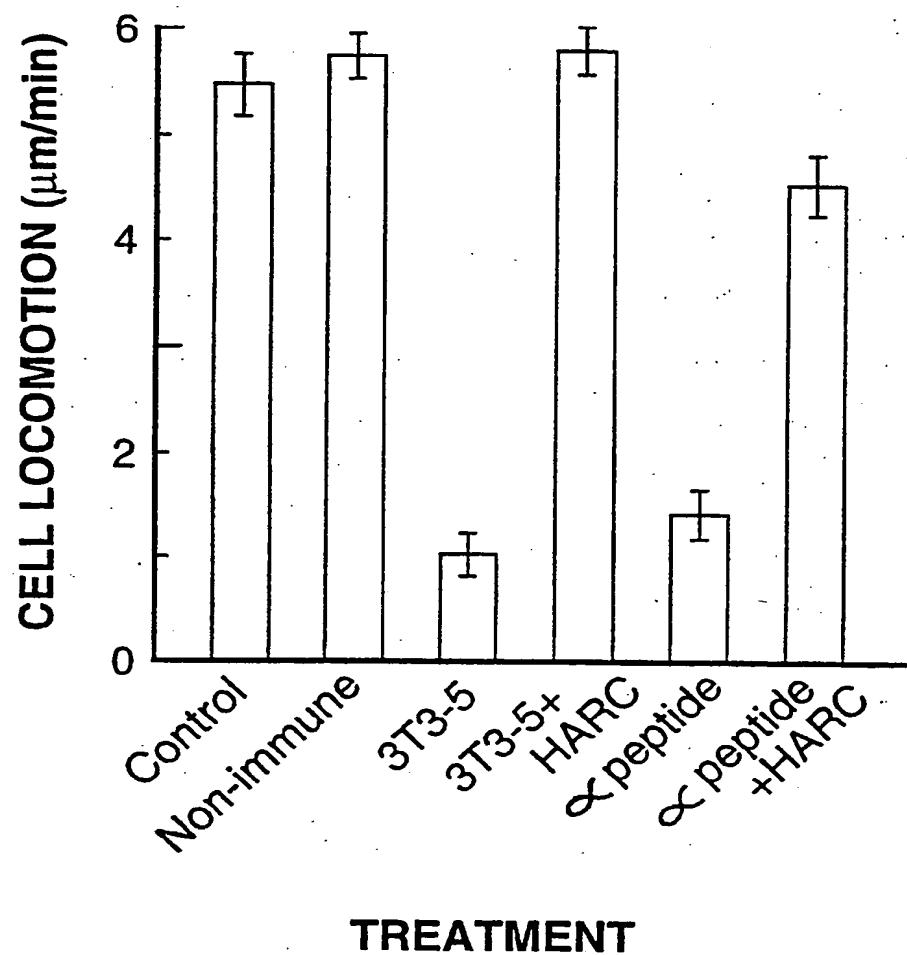


FIG.5.

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5/23

A B C D



FIG.6.

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6/23

A



B **C**



FIG. 7

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7/23

FIG. 8.

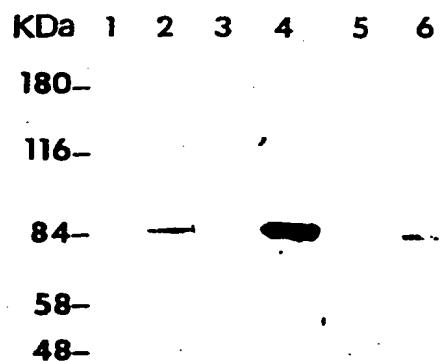
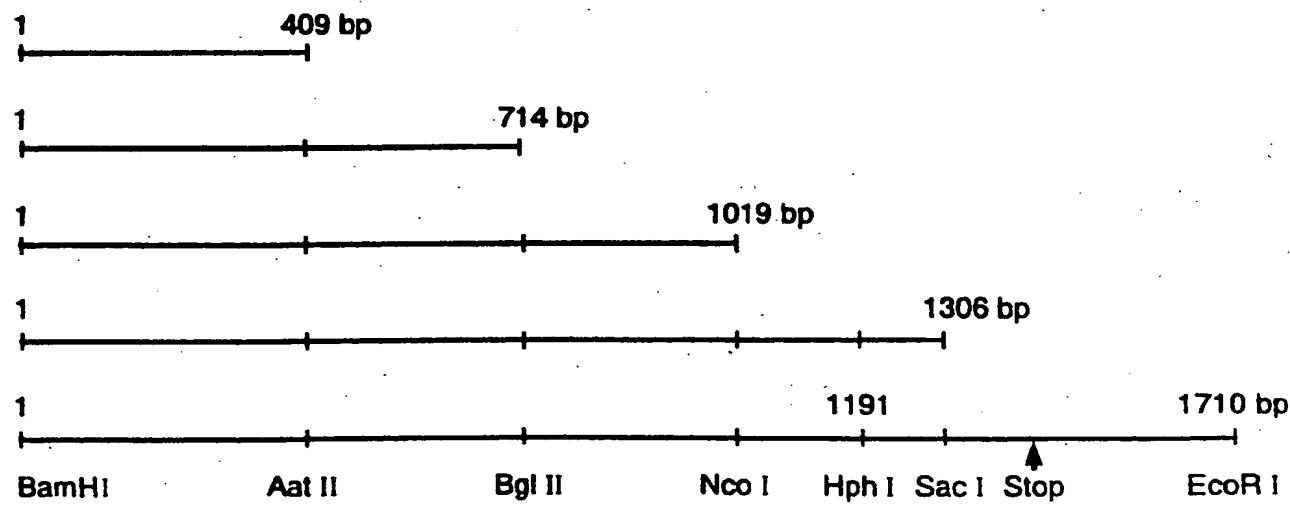


FIG. 9.



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8/23

FIG.10.A.

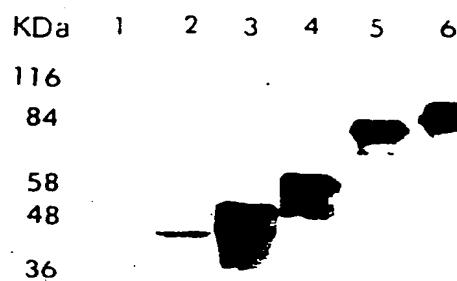
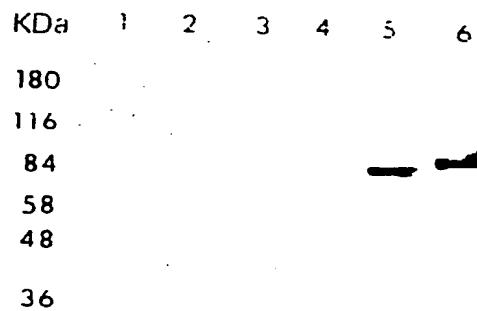


FIG.10.B.



9/23

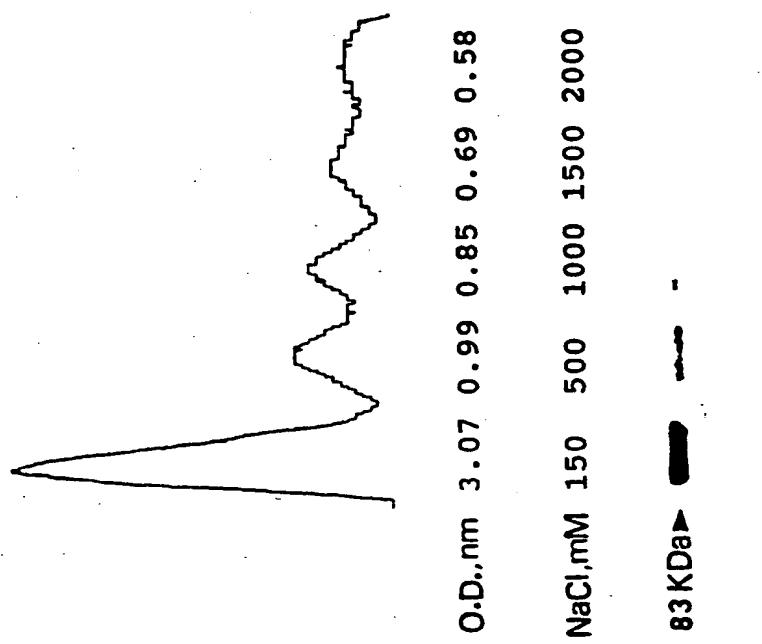


FIG. 11.

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10/23

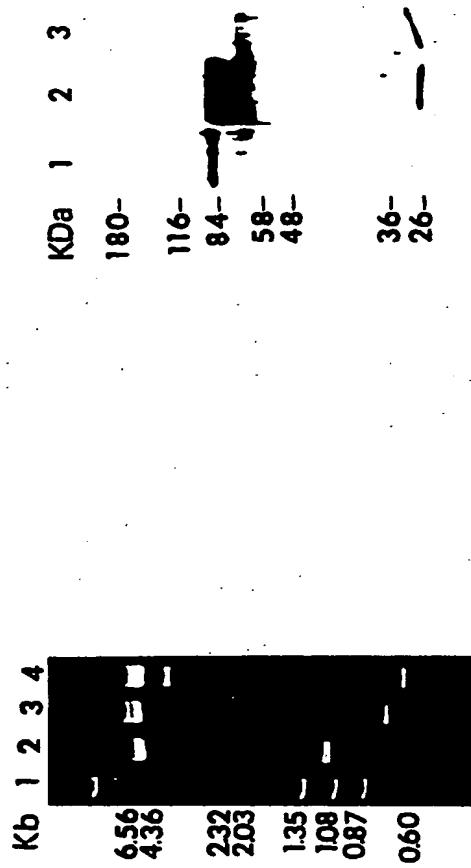


FIG.12A.

FIG.12B.

11/23

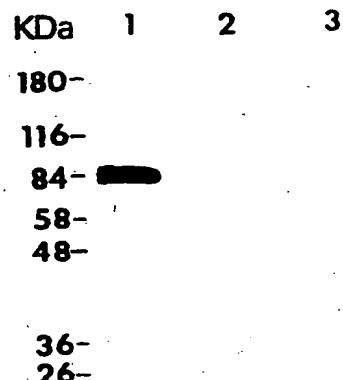


FIG.13A.

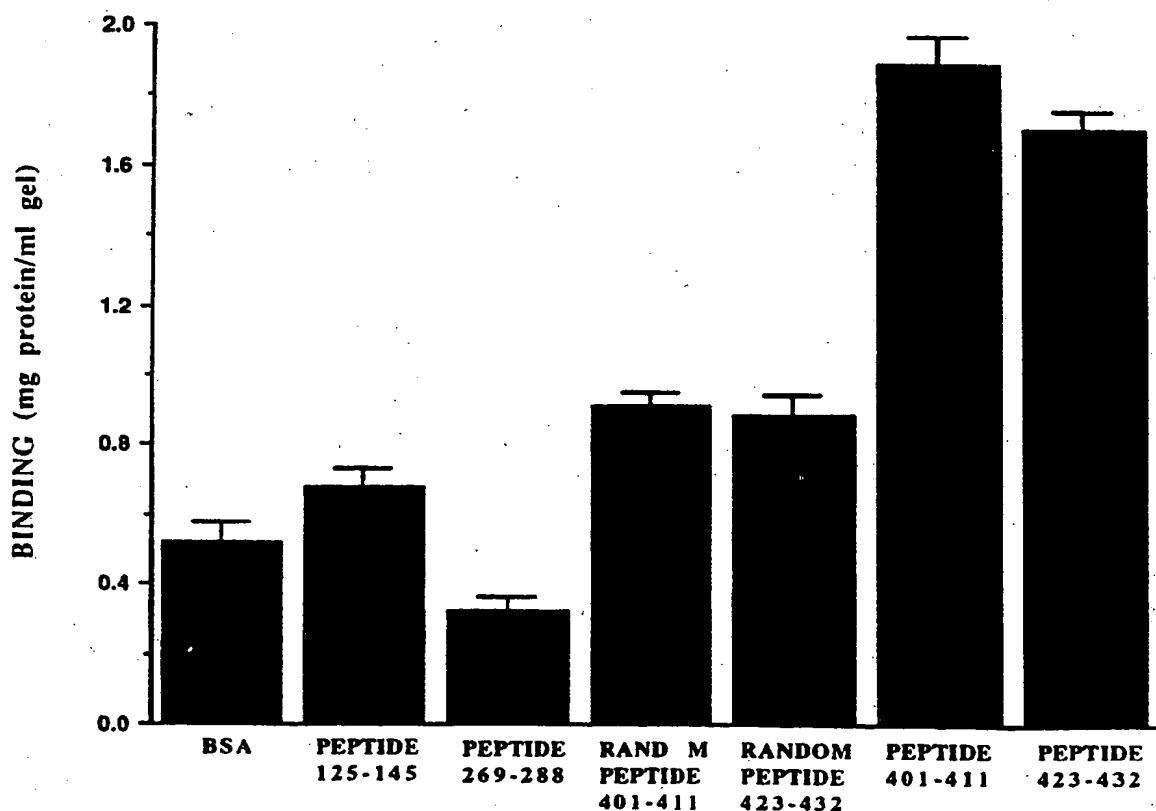


FIG.13.B.

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12/23

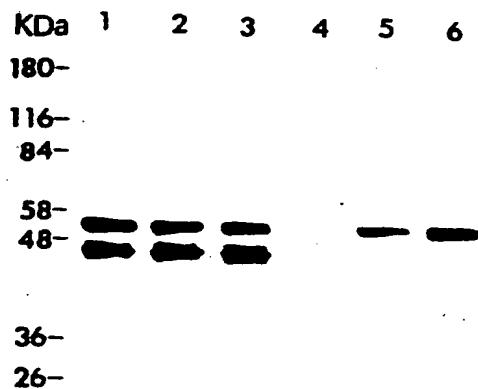
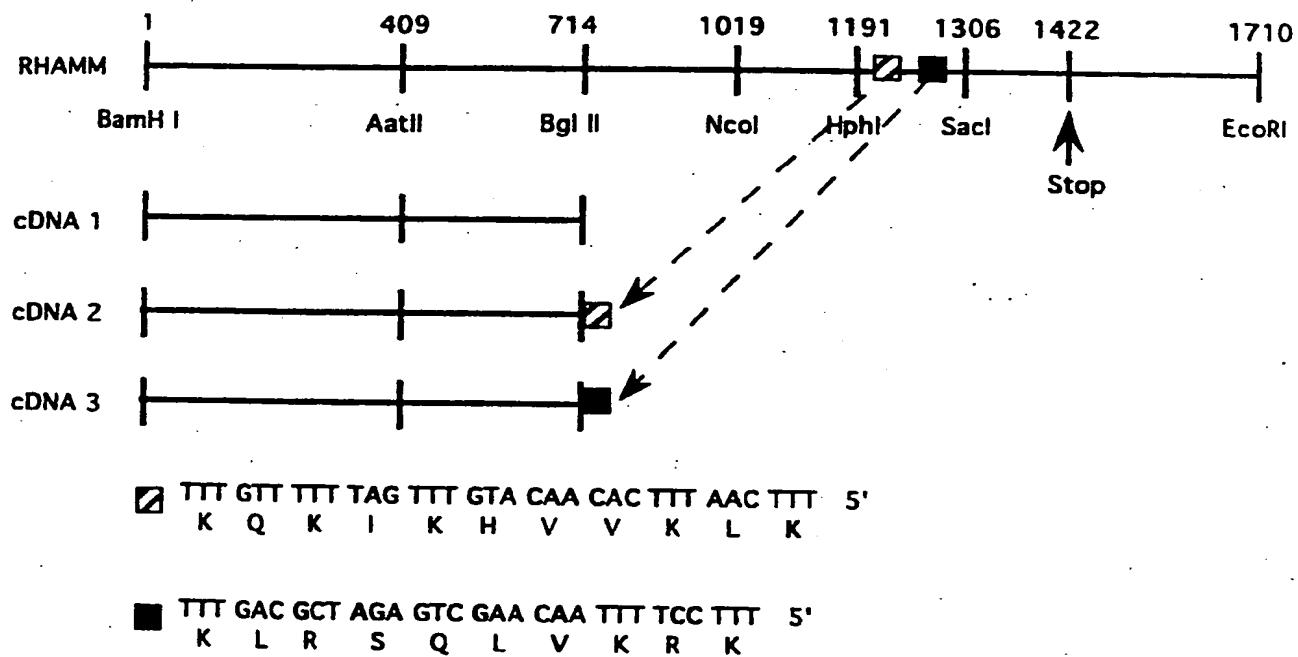


FIG. 14.

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13/23

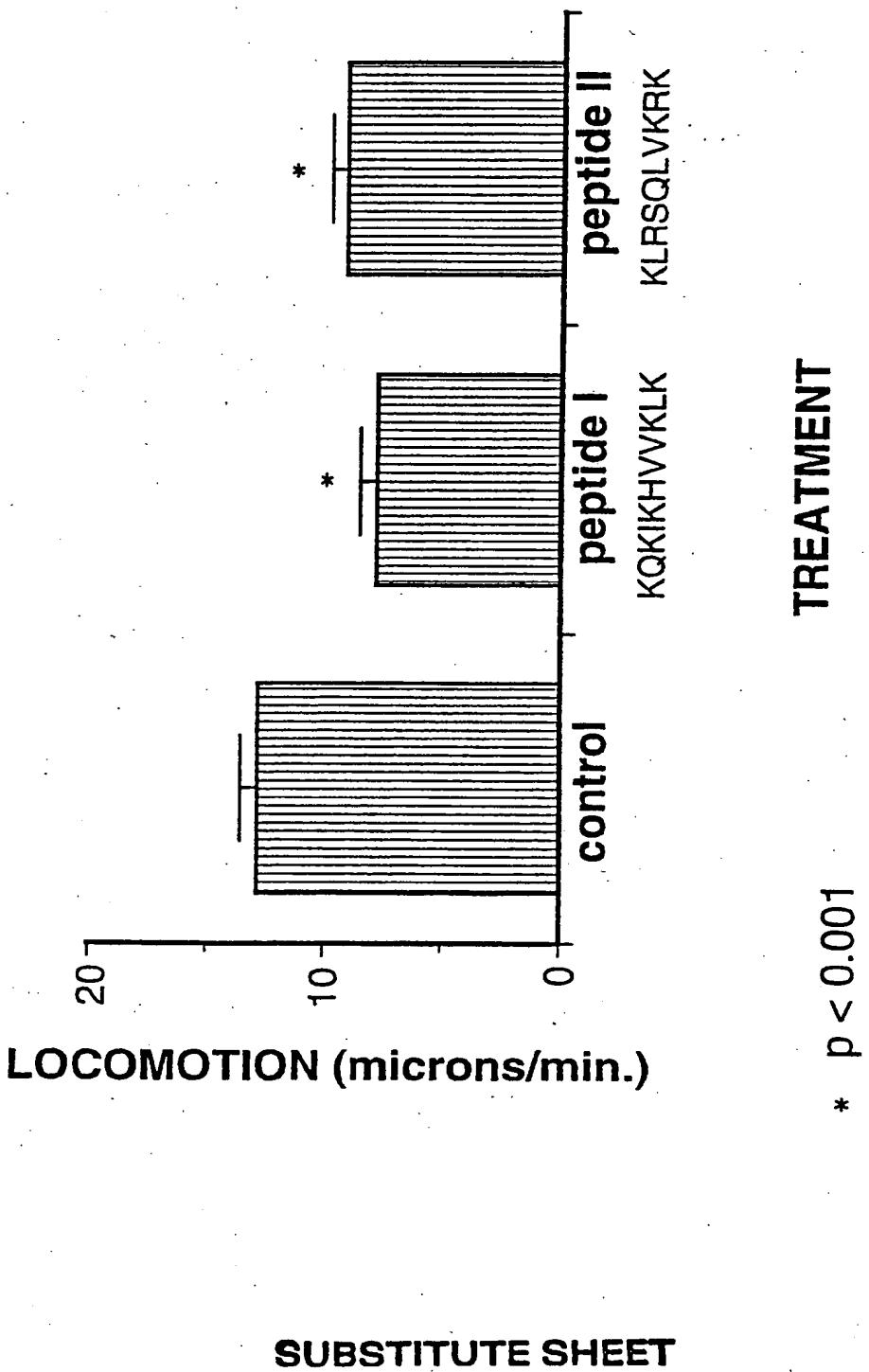


FIG. 15.

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14/23

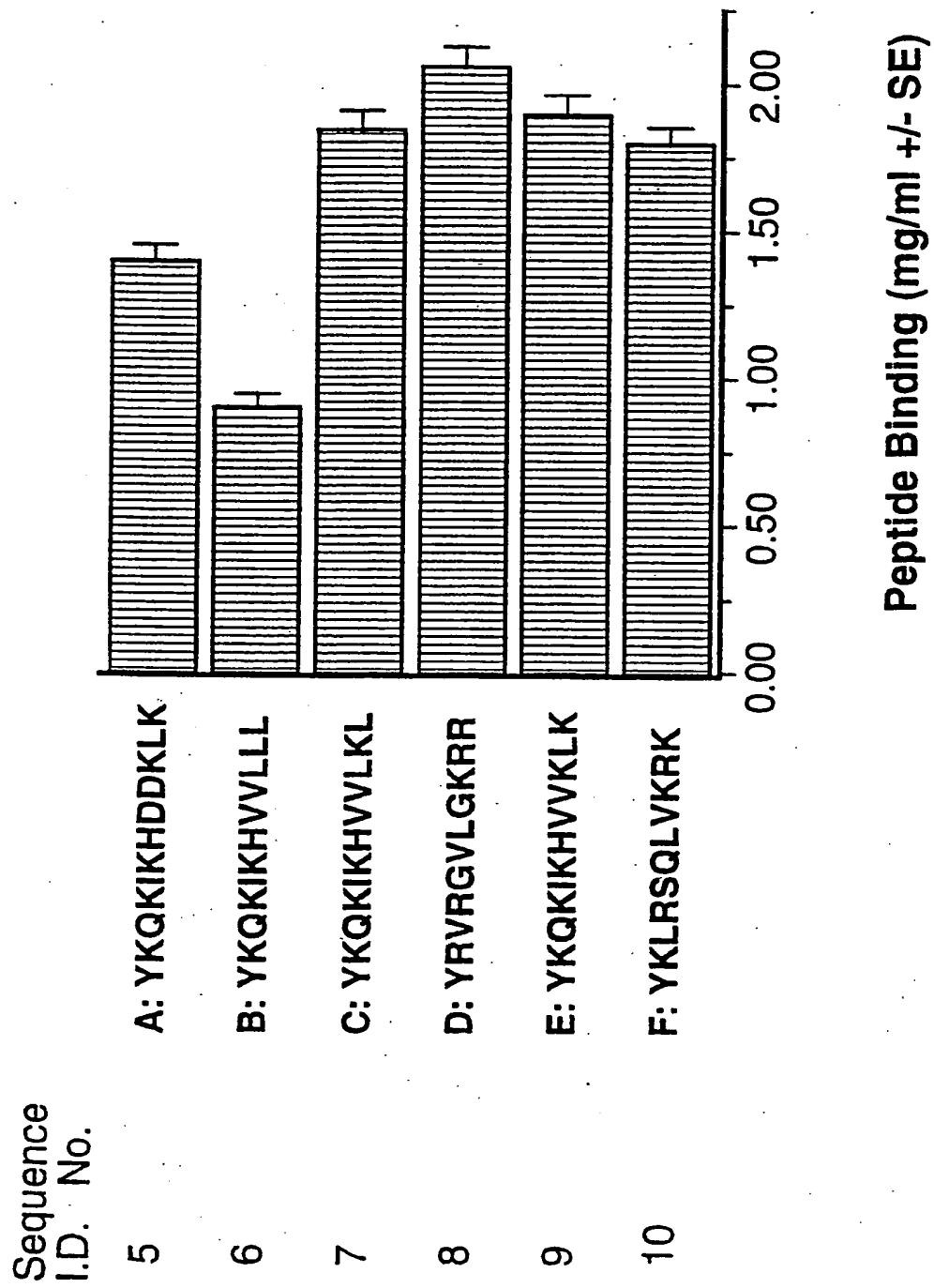


FIG. 16.

15/23

A. Site-directed mutations of peptide 423-432:

! A TTT GAC GCT AGA GTC GAA CAA TTT TCC TTT CTT AAG TAA GTA 5'
 K L R S Q L V K R K EcoRI

I: CAA TTT TCT(TCC) TTT TCTAAG TAA GTA 5'
 V K S (R) K EcoRI

II: GAA CTA(CAA) TTT TCC TTT CTTAAG TAA GTA 5'
 L D (V) K R K EcoRI

III: A TT(A(TT)) GAC GCT AGA GTC GAA CAA TTT TCC TTT CTTAAG TAA GTA 5'
 N (K) L R S Q L V K R K H (R) H (K) EcoRI

IV: GAC GCT AGA GTC GAA CAA GAA GAA GTC(GTC) GTC(TTC) GTC(A(TT)) CTTAAG TAA GTA 5'
 L R S Q L V H (K) H (R) H (K) EcoRI

B. Resultant amino acid sequences:
 Sequence
 ID. No.

Peptide 423-432:	K L R S Q L V K R K	4
Mutation I:	K L R S Q L V K S K	11
Mutation II:	K L R S Q L D K R K	12
Mutation III:	N L R S Q L V K R K	13
Mutation IV:	K L R S Q L V H H H	14

FIG. 17.

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16/23

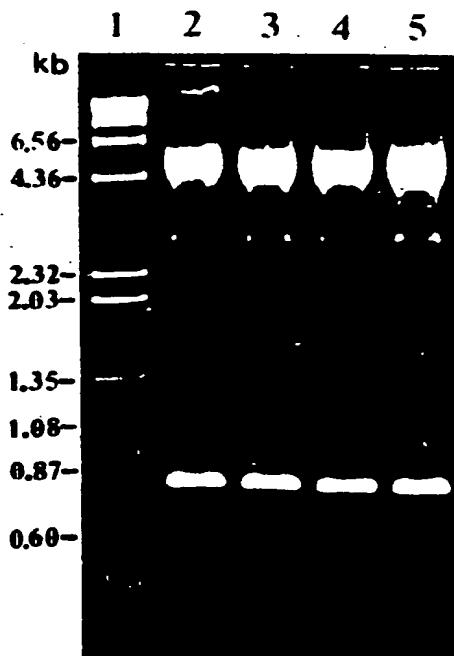


FIG.18A.

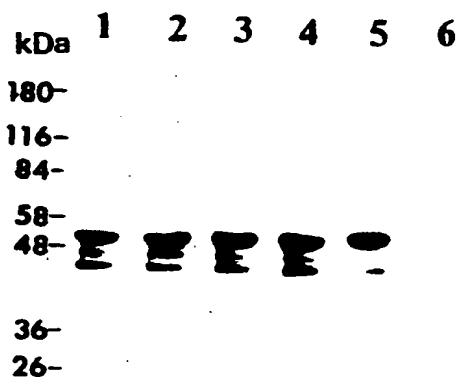


FIG.18.B.

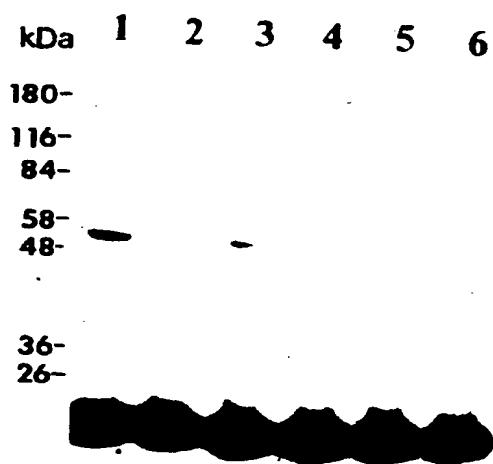


FIG.18.C.

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17/23

RHAMM cDNA



Primer: 3' CGG TAG AAC TAA CGT GTT

GCG ATG GGG TAG AGG TCT GGG TCT TTC GCG
R Y P I S R P R K R

CTT AAG TAA TAA 5'
EcoRI

FIG. 19A.

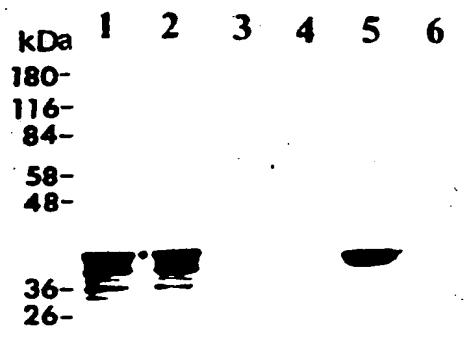
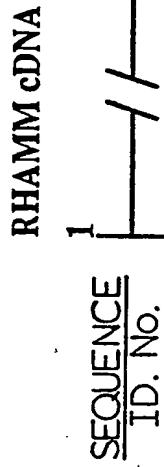


FIG. 19 B.

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18/23

FIG. 20.



16 Primer for CD44 (aa 292-300):

3' CGG TAG AAC TAA CGT GTT
 GCT TCT TCC ACA CCC GTC TTC TTT TTC
 R R C G Q K K K
TTA ACT AAA 5'
EcoRI

17 Primer for Mutation 1:

3' CCC GTC TTC TTT GTC TTA AGT AAA
 G Q K K Q *EcoRI*

18 Primer for Mutation 2:

3' TCC ACA CCC GTC CTC CTT GTC TTA AGT AAA
 R C G Q E E Q *EcoRI*

19/23

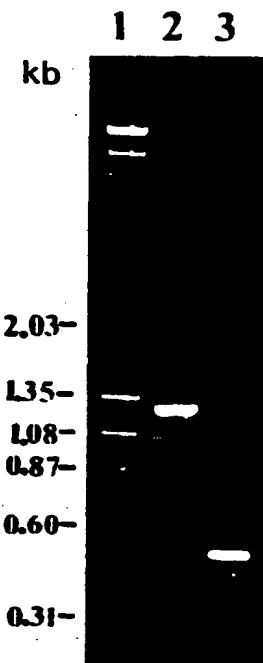


FIG.21A.

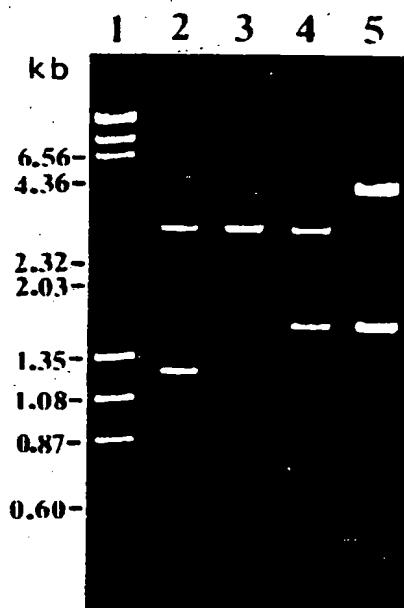


FIG.21B.

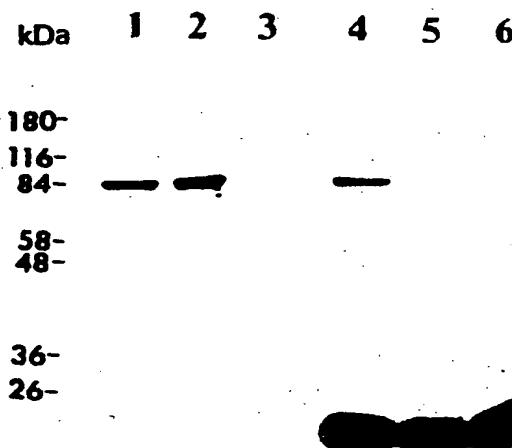


FIG.21 C.

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20/23

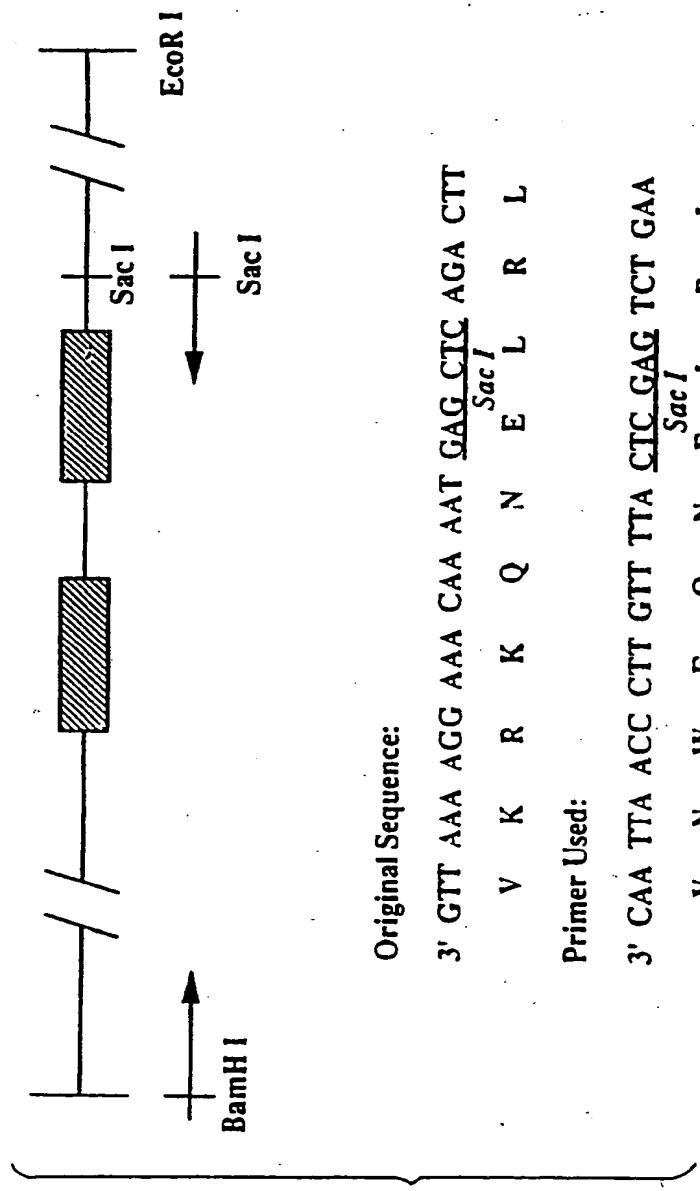


FIG.22.

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<u>MET</u>	<u>Gln</u>	<u>Ile</u>	<u>Leu</u>	<u>Thr</u>	<u>Glu</u>	<u>Arg</u>	<u>Leu</u>	<u>Glu</u>	<u>Gln</u>	<u>Glu</u>	<u>Tyr</u>	<u>Glu</u>	<u>Leu</u>	<u>Gln</u>
ATG	CAG	ATC	CTG	ACA	GAG	AGG	CTG	GCT	CAA	AGG	TAT	GAA	AAG	CAA
Gln	LYS	Glu	Leu	Gln	Ser	Gln	Ser	Leu	Gln	Glu	Leu	Ser	Ala	Arg
CAA	AAA	GAA	TTG	CAA	AGC	CAG	TCA	CTG	CAA	GAG	GAA	CTG	TCT	CGT
Leu	Gln	Gln	Leu	Cys	Ser	Phe	Gln	Glu	MET	Thr	Ser	Glu	Asn	Val
CTG	CAG	CAG	CTC	TGC	TCT	TTC	CAA	GAG	ATG	ACT	TCT	GAG	AAC	Phe
Lys	Glu	Glu	Leu	Lys	Leu	Ala	Glu	Leu	Asp	Ala	Val	Gln	Glu	Glu
AAA	GAA	GAG	CTA	AAG	CTC	GCC	CTG	GAG	TTC	GCG	GTC	CAG	AAG	TTT
Gln	Ser	Glu	Arg	Leu	Val	Lys	Gln	Leu	Glu	Glu	Thr	Lys	Ser	Thr
CAG	AGT	GAA	AGG	CTG	GTT	AAA	CAG	CTG	GAA	GAG	ACG	AAC	TCA	ACT
Leu	Thr	Arg	Leu	Asp	Asn	Leu	Leu	Arg	Glu	Lys	Glu	Val	Glu	Ile
CTG	ACG	CGG	CTG	GAC	AAC	CTG	CTG	AGA	GAG	AAA	GAA	GTC	GAG	CAT
Ala	Arg	His	Ala	Gln	Ala	Ile	Leu	Ile	Ala	Gln	Glu	Tyr	Asn	Asp
GCT	CGT	CMA	GCC	CAA	ATC	TTG	ATT	GCA	CAA	GAG	AAG	TAT	ATT	ATT
Ser	Leu	Arg	Asp	Val	Thr	Ala	Gln	Leu	Glu	Ser	Val	Gln	Lys	Tyr
ACT	CTG	ACG	GTC	GTC	ACT	GCT	ACT	GAG	TTG	GAA	AGT	GTG	CAA	AGG

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FIGURE 23A

Ala	Gln	Ser	Leu	Arg	Asp	Val	Thr	Ala	Gln	Leu	Glu	Gln	Glu	Lys	Tyr	Asn	
GCA	CAG	ACT	CTG	AGG	GAC	GTC	ACT	GCT	CAG	TTC	GAA	AGT	GAG	AAG	TAC	AAT	513
ASP	Thr	Ala	Gln	Ser	Leu	Arg	Asp	Val	Thr	Ala	Gln	Leu	Glu	Ser	Glu	Gln	Lys
GAC	ACA	GCA	CAG	AGT	CTG	AGG	GAC	GTC	ACT	GCT	CAG	TTC	GAA	AGT	GAG	CAA	AAG
TYR	Asn	Asp	Thr	Ala	Gln	Ser	Leu	Arg	Asp	Val	Thr	Ala	Gln	Leu	Glu	Ser	Val
TAC	AAT	GAC	ACA	GCA	CAG	AGT	CTG	AGG	GAC	GTC	ACT	GCT	CAG	TTG	GAA	AGT	GTG
*																	627
Glu	Lys	Tyr	Asn	Asp	Thr	Ala	Gln	Ser	Leu	Arg	Asp	Val	Thr	Ala	Gln	Leu	Ser
GAG	AAG	TAC	AAT	GAC	ACA	GCA	CAG	AGT	CTG	AGG	GAC	GTC	ACT	GCT	CAG	TTG	GAA
TYR	Lys	Ser	Ser	Thr	Leu	Lys	Glu	Ile	Glu	Asp	Leu	Lys	Leu	Glu	Gln	Leu	Ser
TAT	AAG	TCA	TCA	ACA	CTT	AAA	GAA	ATA	GAA	GAT	CTT	AAA	CTG	GAG	AAT	TTG	ACT
Gln	Glu	Lys	Val	Ala	Met	Ala	Glu	Lys	Ser	Val	Glu	Asp	Val	Gln	Gln	Ile	Leu
CAA	GAA	AAA	GAA	GCT	ATG	GCT	GAA	AAA	AGT	GTA	GAA	GAT	GTT	CAA	CAG	CAG	ATA
Thr	Ala	Glu	Ser	Thr	Asn	Gln	Glu	Tyr	Ala	Arg	Met	Val	Gln	Asp	Leu	Gln	Arg
ACA	GCT	GAG	AGC	ACA	AAT	CAA	GAA	TAT	GCA	AGG	ATG	GTT	CAA	GAT	TTG	CAG	AAC
Ser	Thr	Leu	Lys	Glu	Glu	Ile	Lys	Glu	Ile	Thr	Ser	Ser	Phe	Leu	Glu	Lys	Ile
TCA	ACC	TTA	AAA	GAA	GAA	ATT	AAA	GAA	ATT	ACA	TCT	TCA	TTT	CTT	GAG	AAA	ATA
Thr	Asp	Leu	Lys	Asn	Gln	Leu	Arg	Gln	Asp	Glu	Asp	Phe	Arg	Gln	Leu	Glu	
ACT	GAT	TTG	AAA	AAT	CAA	CTC	AGA	CAA	GAT	GAA	GAC	TTT	AGG	AAG	CAG	CTG	GAA
																	969

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FIGURE 23B

Glu	Lys	Gly	Lys	Arg	Thr	Ala	Glu	Lys	Asn	Val	Met	Thr	Glu	Leu	Thr	Met	Glu
GAG	AAA	GGA	AAA	AGA	ACA	GCA	GAG	AAA	AAT	GTA	ATG	ACA	GAA	TTA	ACC	ATG	GAA
Ile	Asn	Lys	Trp	Arg	Leu	Leu	Tyr	Glu	Glu	Tyr	Glu	lys	Pro	Phe	Gln	Gln	
ATT	AAT	AAA	TGG	GCT	CTC	CTA	TAT	GAA	CTA	TAT	GAA	AAA	ACT	CCT	TTT	CAG	CAA
Gln	Leu	Asp	Ala	Phe	Glu	Ala	Glu	Lys	Gln	Ala	Leu	Asn	Glu	Gly	Ala	Thr	
CAA	CTG	GAT	GCC	TTT	GAA	GCC	GAG	AAA	CAG	GCA	TTG	TTG	AAT	GAA	CAT	GGT	GCA
Gln	Glu	Gln	Leu	Asn	Lys	Ile	Arg	Asp	Ser	Tyr	Ala	Gln	Leu	Gly	His	Gln	Asn
CAG	GAG	CAG	CTA	AAT	AAA	ATC	AGA	GAC	TCC	TAT	GCA	CAG	CTA	CTT	GGT	CAC	AAC
Leu	Lys	Gln	Lys	His	Val	Val	Lys	Leu	Lys	Asp	Glu	Asn	Ser	Gln	Leu	Lys	
CTA	AAG	CAA	AAA	ATC	AAA	CAT	GTG	AAA	TTG	AAA	GAT	GAA	AAT	AGC	CAA	CTC	AAA
Ser	Glu	Val	Ser	Lys	Leu	Arg	Ser	Gln	Leu	Val	Lys	Arg	Lys	Gln	Asn	Glu	
TCG	GAG	GTG	TCA	AAA	CTC	CGA	TCT	CAG	CTT	GTT	AAA	AGG	AAA	AAT	GAG	CTC	AGA
Leu	Gln	Gly	Glu	Leu	Asp	Lys	Ala	Leu	Gly	Ile	Arg	His	Phe	Asp	Pro	Ser	
CTT	CAG	GGA	GAA	TTA	GAT	AAA	GCT	CTG	GGC	ATC	AGA	CAC	TTT	GAC	CCT	TCC	AAG
Phe	Cys	His	Ala	Ser	Lys	Glu	Asn	Phe	Thr	Pro	Leu	Lys	Gly	Asn	Pro	Asn	
TTT	TGT	CAT	GCA	TCT	AAG	GAG	AAT	TTT	ACT	CCA	TTA	AAA	GAA	GGC	AAC	CCA	TGC
Cys																	
	TGC		TGA														

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FIGURE 23C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 93/00158

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶			
According to International Patent Classification (IPC) or to both National Classification and IPC			
Int.Cl. 5 C12N15/12; A61K37/02;		C07K13/00; A61K39/395;	C07K7/06; G01N33/68;
		C12P21/08 C12N1/21	
II. FIELDS SEARCHED			
Minimum Documentation Searched ⁷			
Classification System		Classification Symbols	
Int.Cl. 5		C12N ;	C07K ;
		A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸			
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹			
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²		Relevant to Claim No. ¹³
X	THE JOURNAL OF CELL BIOLOGY vol. 112, no. 5, March 1991, pages 1041 - 1047 TURLEY, E.A. ET AL.; 'Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of Ras-transformed cells.' cited in the application see the whole document		1,4,7, 13,14, 22,25, 28, 30-34, 36-44 -/-
"Special categories of cited documents : ¹⁰			
"A" document defining the general state of the art which is not considered to be of particular relevance			
"E" earlier document but published on or after the international filing date			
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)			
"O" document referring to an oral disclosure, use, exhibition or other means			
"P" document published prior to the international filing date but later than the priority date claimed			
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention			
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step			
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art			
"A" document member of the same patent family			
IV. CERTIFICATION			
Date of the Actual Completion of the International Search 23 AUGUST 1993		Date of Mailing of this International Search Report 08-09-1993	
International Searching Authority EURPEAN PATENT OFFICE		Signature of Authorized Officer NAUCHE S.A.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>DEVELOPMENTAL BIOLOGY vol. 146, 1991, pages 186 - 197 BARNEJEE, S.D. ET AL.; 'Monoclonal antibody to chick embryo hyaluronan-binding protein : Changes in distribution of binding protein during early brain development.' See Introduction, see page 187, column 2, line 5 - page 188, column 2, line 5 page 189, Paragraph "Results" ---</p>	1,4,7, 13,14, 22,25, 28, 30-34, 36-44
X	<p>BIOCHEMISTRY. vol. 26, 1987, EASTON, PA US pages 2997 - 3005 TURLEY, E.A. ET AL. 'Characterization of Hyaluronate binding proteins isolated from 3T3 and murine sarcoma virus transformed 3T3 cells' cited in the application see the whole document ---</p>	1,4,7, 13,14, 22,25, 30-34, 36-44
P,X	<p>THE JOURNAL OF CELL BIOLOGY vol. 117, no. 6, June 1992, pages 1343 - 1350 HARDWICK C;HOARE K;OWENS R;HOHN HP;HOOK M;MOORE D;CRIPPS V;AUSTEN L;NANCE DM;TURLEY EA; 'Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility' see the whole document ---</p>	1-44
X	<p>THE JOURNAL OF CELL BIOLOGY vol. 105, 1987, NEW YORK, USA pages 2403 - 2408 GOETINCK P.F. ET AL. 'The tandemly repeated sequences of cartilage link protein contain the sites for interaction with hyaluronic acid' see page 2404, column 1, line 8 - page 2404, column 1, line 37 ---</p>	11
A	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 262, no. 36, 1987, BALTIMORE US pages 17757 - 17767 DOEGE, K. ET AL.; 'Complete primary structure of the rat cartilage proteoglycan core protein deduced from cDNA clones' ---</p>	12-18
A		-/-

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>BIOCHEMICA ET BIOPHYSICA ACTA vol. 1078, no. 1, 30 May 1991, AMSTERDAM, NL pages 12 - 18 FORSSBERG N;GUSTAFSON S; 'Characterization and purification of the hyaluronan-receptor on liver endothelial cells.'</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA93/00158

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 30,31,33,35,36,38-46 are directed to a method of treatment of the human/animal body (Rule 39.1(iv) PCT) the search has been carried out and based on the alleged effects of the compound/composition
2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

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1/23

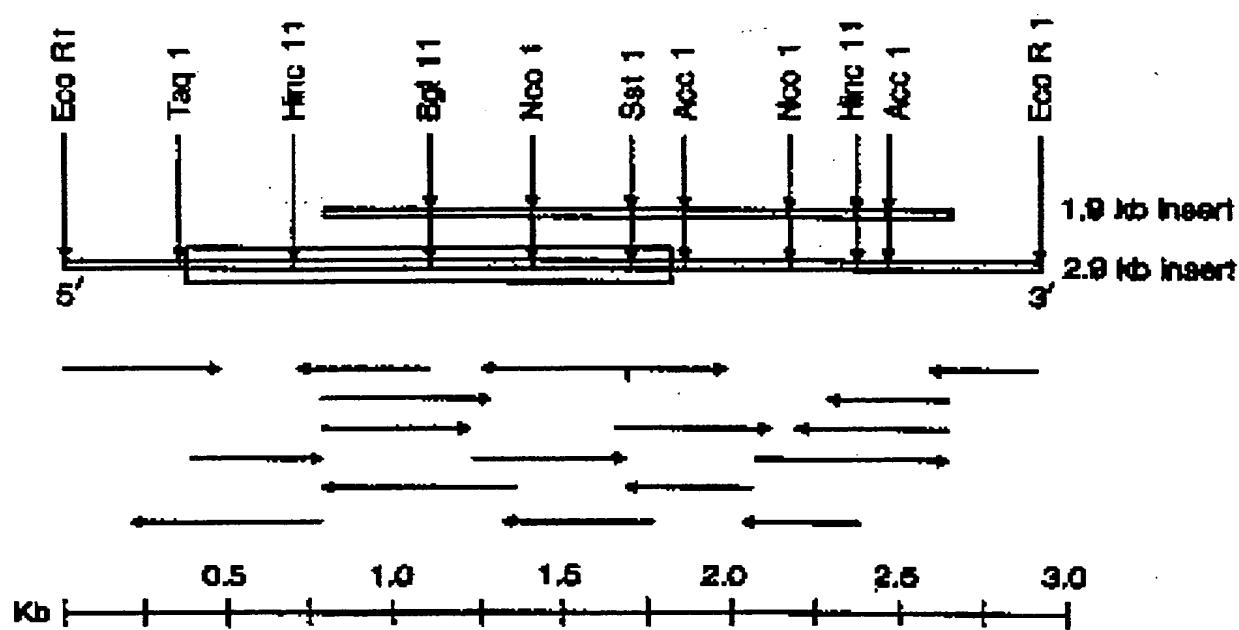


FIGURE 1

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2/23



FIG.2a.

FIG.2b.

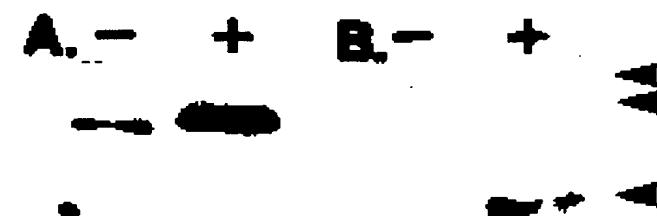


FIG.3a.



FIG.3b.

3/23



C. - + D. - +



FIG.4.

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4/23

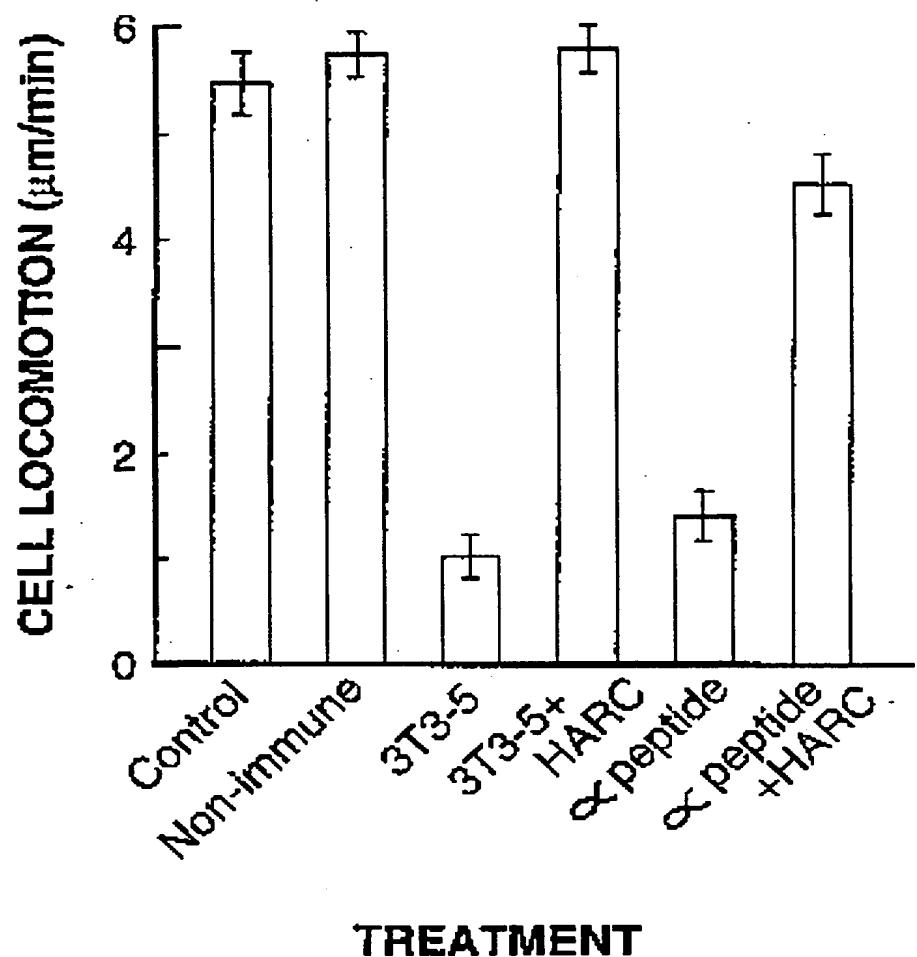


FIG. 5.

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5/23

A B C D



FIG. 6.

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6/23

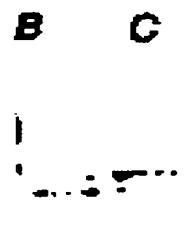
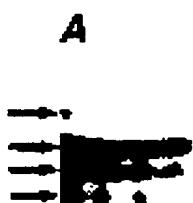


FIG.7

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7/23

FIG. 8.

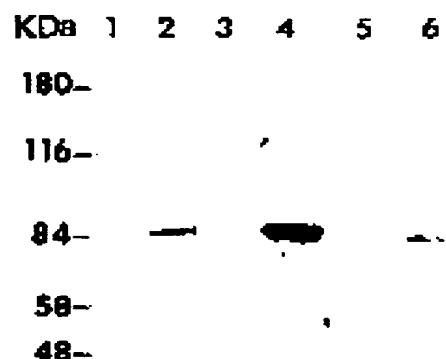
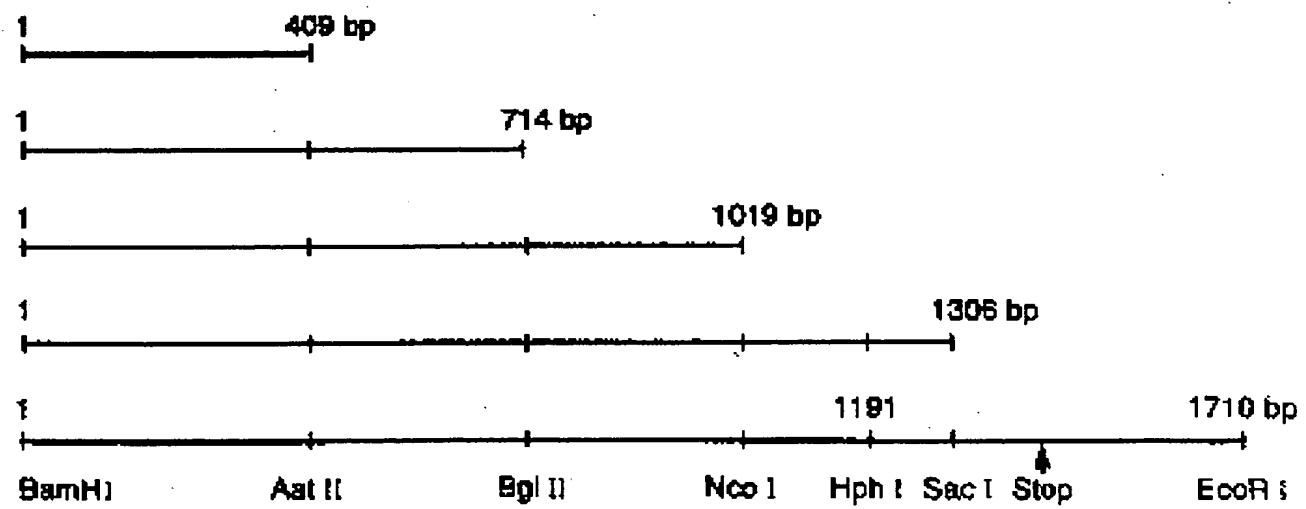


FIG. 9.



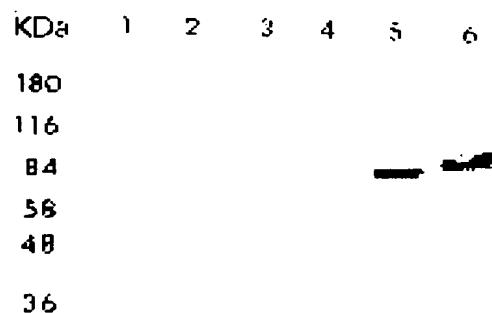
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8/23

FIG.10A.



FIG.10.B.



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9/23

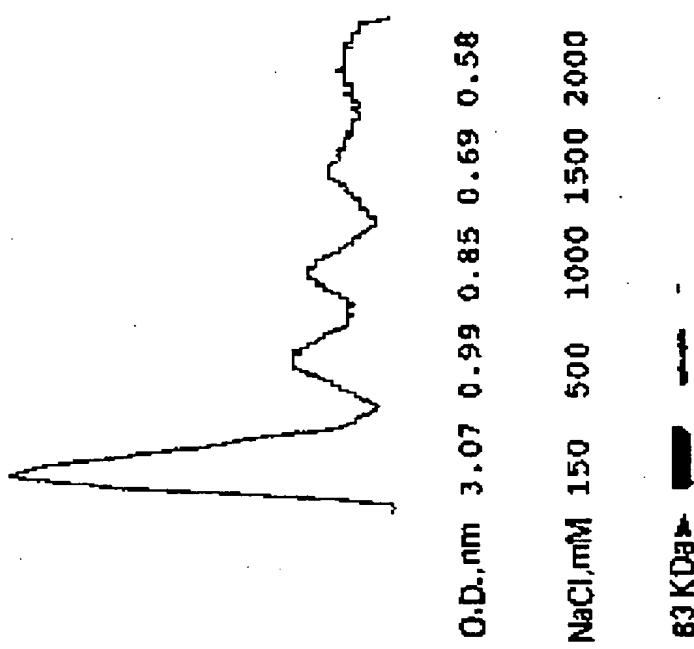


FIG. 11.

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10/23

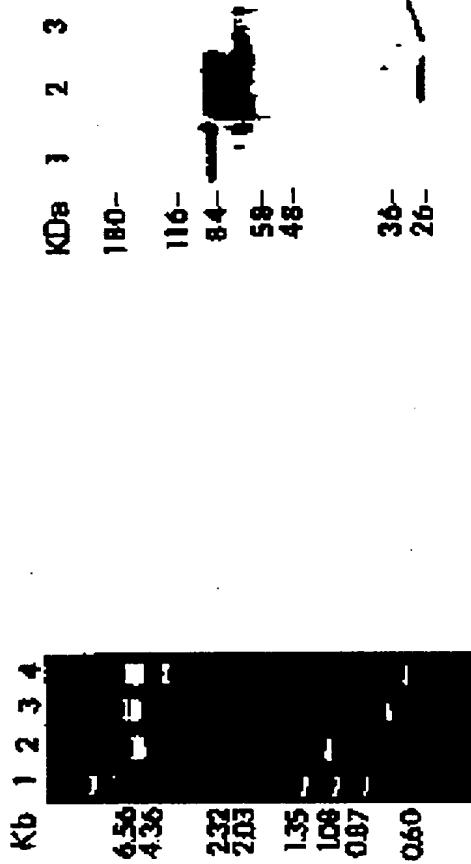


FIG 12.B.

FIG 12.A.

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11/23

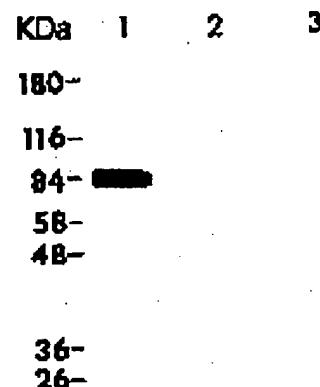


FIG. 13A.

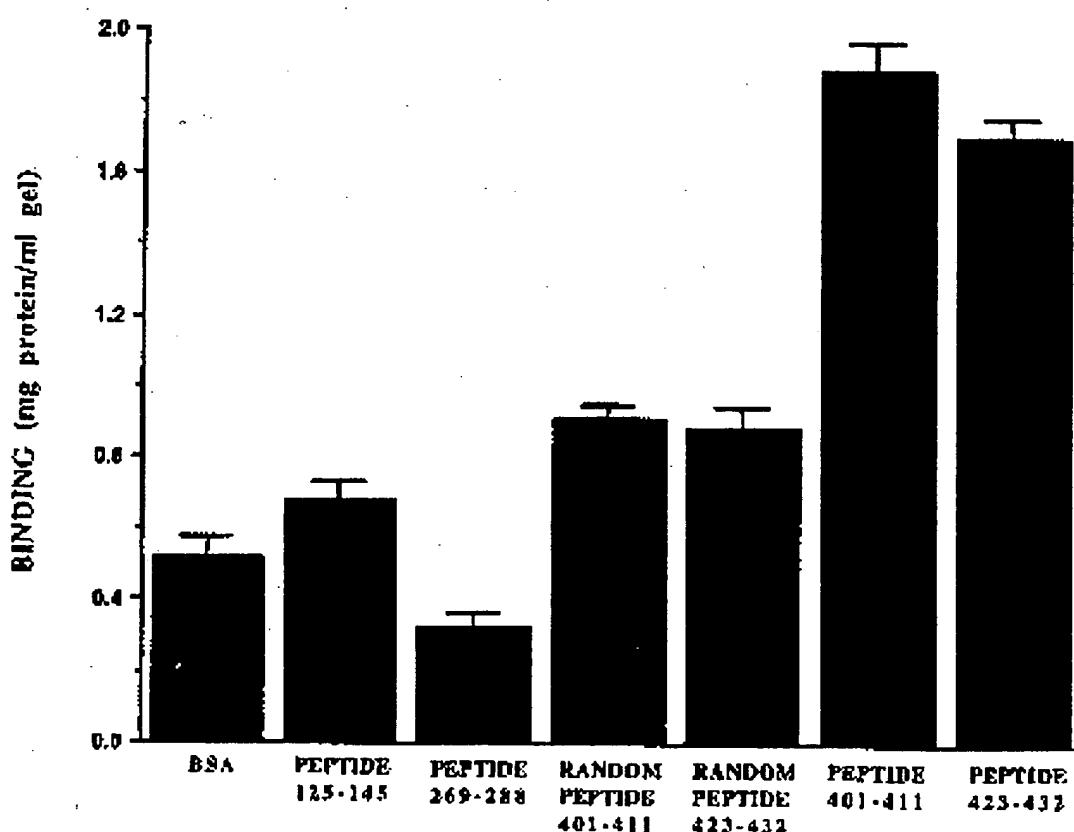
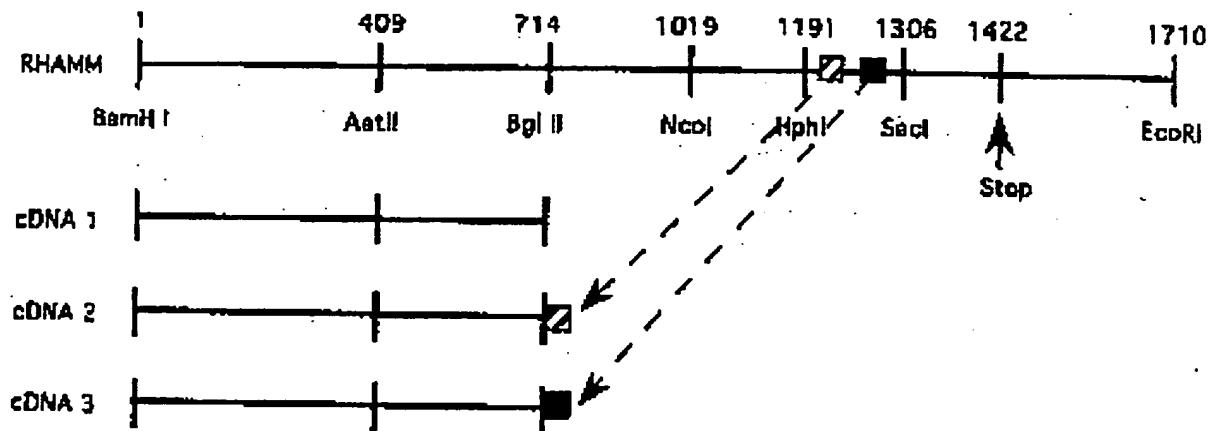


FIG. 13.B.

12/23



■ TTT GTT TTT TAG TTT GTA CAA CAC TTT AAC TTT 5'
 K Q K I K H V V K L K

■ TTT GAC GCT AGA GTC GAA CAA TTT TCC TTT 5'
 K L R S Q L V K R K

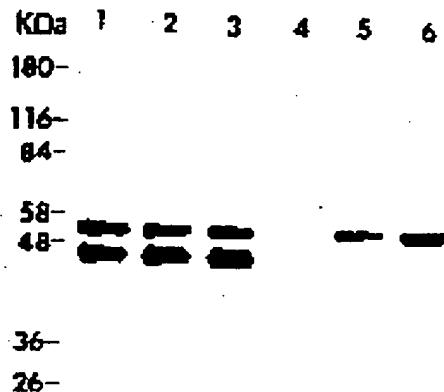


FIG. 14

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13/23

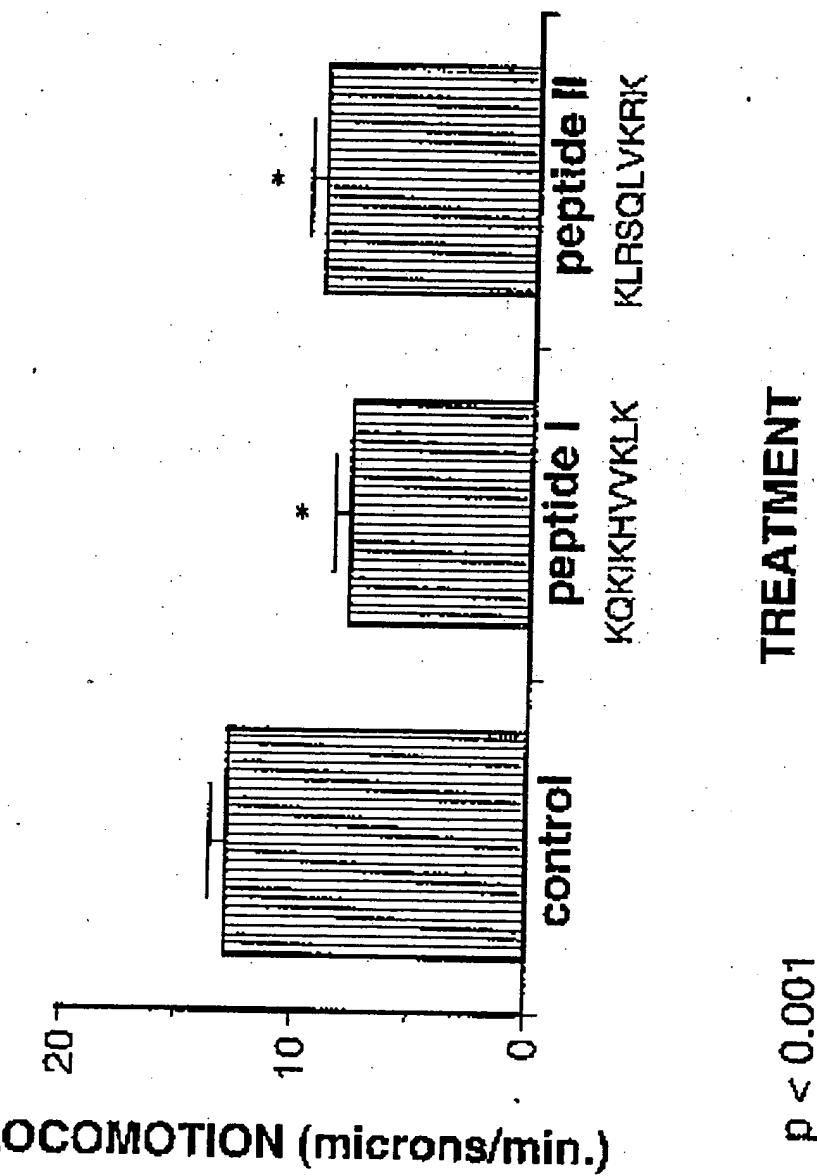
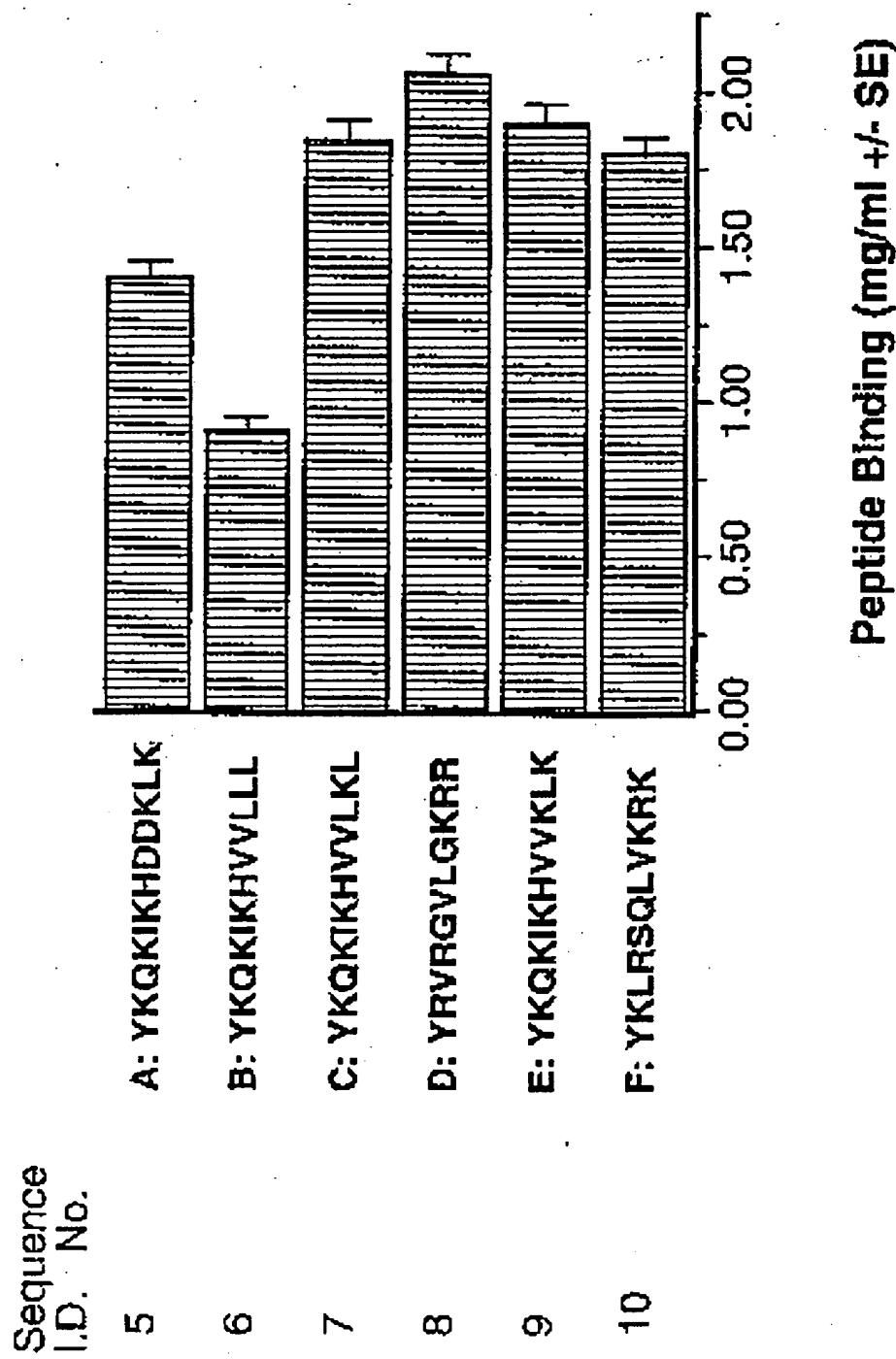


FIG. 15.

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14/23

FIG. 16.



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15/23

A. Site-directed mutations of peptide 423-432:

1:

I: A TTT GAC GCT AGA GTC GAA CAA TTT TCC TTT CTT AAC TAA GTC 5'
 K R S (R) K EcoRI

II: GAA CTA CAA TTT TCC TTT CTT AAC TAA GTC 5'
 L D (R) K R S (R) K EcoRI
 N (R) L R S Q L V K R K EcoRI

III: GAC GCT AGA GTC GAA CAA GTC (GTC) GTC (GTC) GTC (GTC) CTT AAC TAA GTC 5'
 E (R) S Q L V H (R) H (R) R (K) EcoRI

B. Resultant amino acid sequences:
 Peptide 423-432: K L R S Q L V K R K
 Mutation I: K L R S Q L V K S K 4
 Mutation II: K L R S Q L D K R K 11
 Mutation III: N L R S Q L V K R K 12
 Mutation IV: K L R S Q L V H H R 13

sequence
 ID No.
 4
 11
 12
 13
 14

FIG. 17

16/23

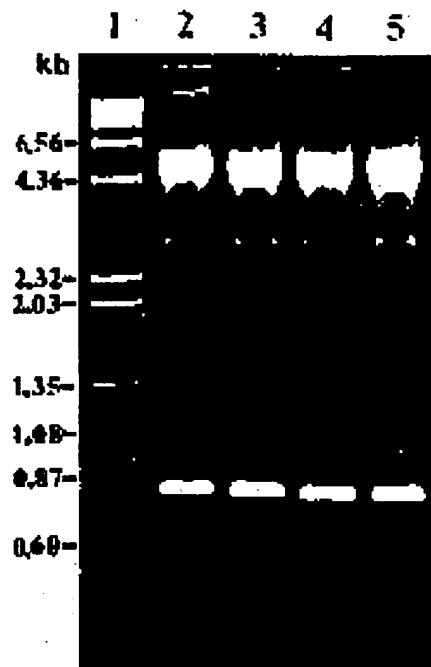


FIG.18A.

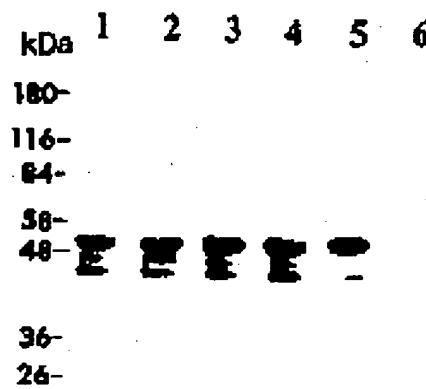


FIG.18.B.

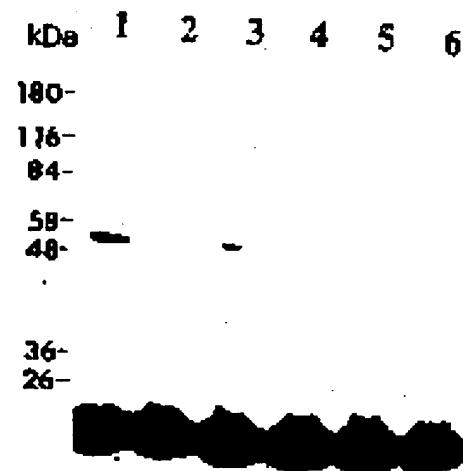


FIG.18.C.

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17/23

RHAMM cDNA



Primer: 3' CGG TAG AAC TAA CGT GTT

GCG ATG GGG TAG AGG TCT GGG TCT TTC GCG
R Y P I S R P R K R
CTT AAG TAA TAA 5'
EcoRI

FIG.19A.

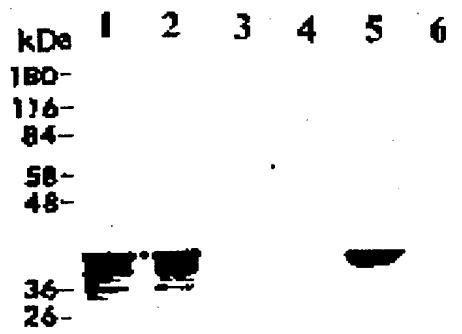


FIG.19B.

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18/23

FIG. 20

RHAMM cDNA

Primer for CD44 (aa 292-300):

Primer for Mutation 1:

3' CCC GTC TTC TTT GTC TAA ACT AAA
G Q K K Q EcoRI

Primer for Mutation 2:

3' TCC ACA CCC GTC CTC CTT GTC TTA AGT AAA
 R C G Q E E Q ECDRI

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19/23

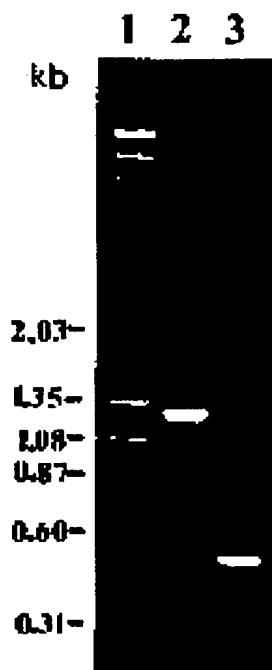


FIG. 21A.

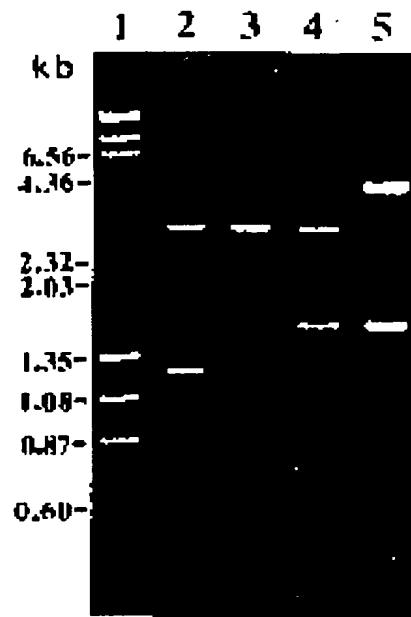


FIG. 21B.

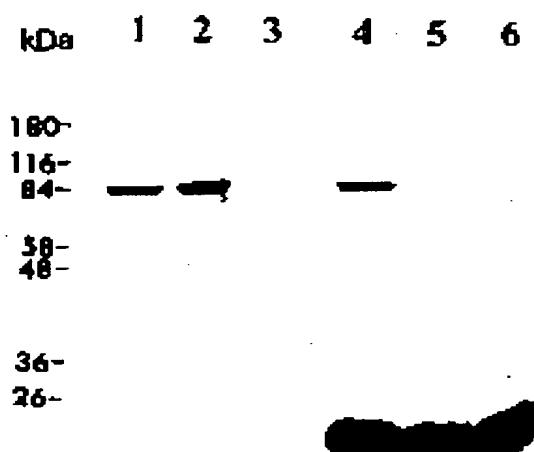


FIG. 21 C.

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20/23

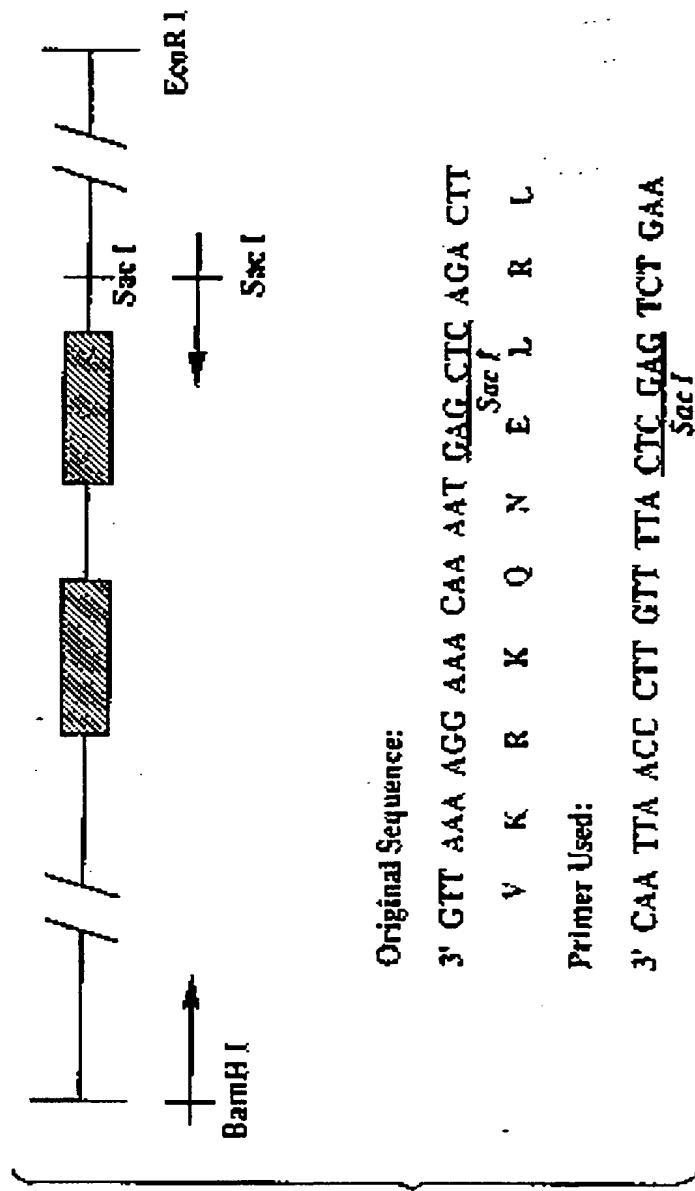


FIG.22.

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Met	Gln	Ile	Leu	Thr	Glw	Arg	Leu	Glu	Gln	Glu	Arg	Gln	Glu	Tyr	Glu	Lys	Leu	Gln
ATG	CAG	ATC	CTG	ACA	CAG	AGC	CTG	GCT	GAA	AGG	CAA	GAA	TAT	GAA	AAG	CTG	CAA	ATG
Gln	Lys	Glu	Leu	Gln	Ser	Gln	Ser	Leu	Gln	Gln	Gln	Gln	Glu	Glu	Leu	Ser	Ala	Arg
CAG	AAA	GAA	TTG	CAA	AGC	CAC	TCA	CTG	CAC	CAC	CAC	CAC	GAG	AGC	TCT	GCT	CGT	TTA
Leu	Gln	Gln	Gln	Leu	Cys	Ser	Phe	Gln	Glu	Glu	Met	Thr	Ser	Glu	Lys	Asn	Val	Phe
CTG	CAG	CAG	CAG	CTC	TGC	TCT	TTC	CAG	GAA	GAA	ATG	ACT	TCT	GAG	AGC	MAC	GTC	TCT
Lys	Glu	Glu	Glu	Leu	Lys	Leu	Ala	Glu	Leu	Asp	Ala	Val	Gln	Gln	Lys	Glu	Glu	Glu
AAA	GAA	GAG	GAG	CTA	AGC	CTC	GCC	CTG	CTG	TTC	GAT	GGG	CTC	CAG	ATG	GAG	GAG	CTG
Gln	Ser	Glu	Glu	Arg	Leu	Val	Lys	Gln	Leu	Glu	Glu	Glu	Tyr	Lys	Ser	Thr	Ala	Glu
CAG	AGT	GAA	GAG	CTG	GTG	AAA	CAG	CTG	GAA	CAC	GAA	GAA	AGC	AGC	TCA	ACT	GCA	GAA
Leu	Thr	Arg	Leu	Asp	Asn	Leu	Arg	Glu	Lys	Glu	Val	Glu	Leu	Glu	Lys	His	Ile	
CTG	ACG	CGG	CTG	GAC	AGC	ATC	CTG	CTG	AGA	AGA	GAA	GAA	GTT	CAA	CTG	GAG	AAA	ATG
Ala	Arg	His	Ala	Gln	Ala	Ile	Ile	Leu	Ala	Gln	Glu	Lys	Tyr	Asn	Asp	Thr	Ala	Gln
GCT	CGT	CAA	GCC	CAA	GAA	ATC	TTC	ATG	ATP	CCA	CAC	CAC	AGC	TAT	ATT	GAC	GCA	CAG
Ser	Leu	Arg	Asp	Val	Thr	Ala	Gln	Leu	Glu	Ser	Val	Gln	Glu	Lys	Tyr	Asn	Asp	Thr
AGT	CTG	AGG	GTC	ACT	GTC	ACT	GCT	GAG	TTC	GAA	AGT	GTC	CAA	GAC	AAC	TAC	ATG	ACG

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FIGURE 21A

Ala	Gln	Ser	Ileu	Arg	Asp	Val	Thr	Ala	Gln	Ileu	Glu	Ser	Glu	Ileu	Glu	Lys	Tyr	Asn
GCA	CAG	AGT	CTG	AGG	GAC	GTC	ACT	GCT	CAG	TTC	GAA	AGT	GAG	CAG	GAC	TAC	TAC	AT
Asp	Thr	Ala	Gln	Ser	Ileu	Arg	Asp	Val	Thr	Ala	Gln	Ileu	Glu	Ser	Glu	Gln	Glu	Lys
GAC	ACA	GCA	CAG	AGT	CTG	AGG	GAC	GTC	ACT	GCT	CAG	TTC	GAA	AGT	SAG	CAG	GAG	AGC
*	TYR	Asn	Asp	Thr	Ala	Gln	Ser	Ileu	Arg	Asp	Val	Thr	Ala	Gln	Ileu	Glu	Ser	Val
TAC	AAT	GAC	ACA	CCA	CAG	AGT	CTG	AGG	GAC	GTC	ACT	GCT	CAG	TTC	GAA	AGT	GTG	CAG
*	Glu	Lys	Tyr	Asn	Asp	Thr	Ala	Gln	Ser	Ileu	Arg	Asp	Val	Thr	Ala	Gln	Ileu	Glu
GAG	AAG	TAC	AAT	GAC	ACA	CCA	CAG	AGT	CTG	AGG	GAC	GTC	ACT	GCT	CAG	TTG	GAA	AGC
Tyr	Lys	Ser	Ser	Thr	Ileu	Lys	Glu	Ile	Glu	Asp	Ileu	Lys	Leu	Glu	Asn	Ileu	Thr	Ileu
TAT	AAG	TCA	TCA	ACA	CTT	AAA	GAA	ATA	GAA	GAT	CCT	AAA	CTG	GAG	AAT	TTG	ACT	CTA
Gln	Glu	Lys	Val	Ala	Met	Ala	Glu	Lys	Ser	Val	Glu	Asp	Val	Gln	Gln	Ile	Ileu	Leu
CAA	GAA	AAA	GTA	GCT	ATG	GAA	AAA	AAA	AGT	GTA	GAA	GAT	GTT	CAG	CAG	CAG	ATA	TTG
Thr	Ala	Glu	Ser	Thr	Asn	Gln	Glu	Ileu	Tyr	Ala	Arg	Met	Val	Gln	Asp	Ileu	Gln	Arg
ACA	GCT	GAG	AGC	ACA	AAT	CAA	GAA	TCT	CCA	AGG	ATG	GTT	CAG	GAT	TTG	CAG	AAC	AGA
SER	THR	ILEU	LYS	GLU	GLU	ILE	LYS	GLU	ILE	THR	SER	SER	PHE	ILEU	GLU	LYS	ILEU	ALA
TCA	ACC	TTC	AAA	GAA	GAA	ATT	AAA	GAA	ATC	ACA	TCT	TCA	TTT	CAG	AAA	ATA	TTT	ATA
Thr	Asp	Leu	Lys	Asn	Gln	Ileu	Arg	Gln	Gln	Asp	Glu	Asp	Phe	Arg	Lys	Gln	Leu	Glu
ACT	GAT	TTC	AAA	AAT	CAA	CTC	AGA	CAA	CAA	GAT	GAA	GAC	TTT	AGG	AAG	CAG	CTG	GAA

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FIGURE 2B

Glu	Lys	GLY	Lys	Arg	Thr	Ala	Glu	Lys	Glu	Asn	Val	Met	Glu	Leu	Thr	Glu	Leu	Thr	Met	
GAC	AAA	GGA	AAA	AGA	ACA	GCA	GAG	AAA	GAA	ATT	GTA	ATC	ACA	GAA	TTA	ACC	ATC	ATC	GAA	1026
Ile	Asn	Lys	TER	Arg	Leu	Tyr	Glu	Glu	Tyr	Glu	Thr	Lys	Pro	Phe	Gln	Gln	CAC	CAC	1083	
ATT	ATT	AAA	TGG	GCT	CTC	TAT	GAA	CTA	TAT	GAA	AAK	ACT	AAA	CCP	TTT	CAC	CAC	CAC	1083	
Gln	Leu	Asp	Ala	Phe	Glu	Ala	Glu	Lys	Gln	Ala	Leu	Leu	Asn	Glu	His	Gly	Ala	Thr	1140	
CAA	CTG	GAT	GCC	TTT	GAA	GCC	GAG	AAA	CAG	GCA	TTG	TTG	ATG	GAA	CAT	GGT	GCA	ACT	1140	
Gln	GLU	GLN	Leu	Asn	Lys	Ile	Arg	Asp	Ser	Tyr	Ala	Gln	Leu	Gly	His	Gln	Asn	Asn	1197	
CAG	GAC	CAG	CTA	ATT	AAA	ATC	AGA	GAC	TCC	TAT	GCA	CAG	CTA	CTT	GGT	CAC	CAC	AAC	1197	
Ile	Lys	GLN	lys	Ile	Lys	His	Val	Val	Lys	Leu	Lys	Asp	Glu	Asn	Ser	Gln	Leu	Lys	23/23	
CTA	AAG	CAA	AAA	ATC	AAA	CAT	CTT	CTG	AAA	TTG	AAA	GAT	GAA	AAT	AGC	CAC	CTC	AAA	12543	
Ser	GlU	val	ser	Lys	Leu	Arg	Ser	Gln	Leu	Val	Lys	Arg	Lys	Gln	Asn	Glu	Leu	Arg	1111	
TCG	CAG	GTG	TCA	AAA	CTC	CGA	TCT	CAG	CAG	CTT	AAA	AGG	AAA	CAA	AAT	GAG	CTC	AGA	1111	
Leu	Gln	GLY	Glu	Leu	Asp	Lys	Ala	Leu	Gly	Ile	Arg	His	Phe	Asp	Pro	Ser	lys	Ala	1368	
CTT	CAG	GGA	GAA	TTA	GAT	AAA	GCT	CTG	GGC	ATC	AGA	CAC	TTT	GAC	CCT	TCC	AAG	GCT	1368	
Phe	Cys	His	Ala	Ser	Lys	Glu	Asn	Phe	Thr	Pro	Leu	lys	Glu	Gly	Asn	Pro	Asn	CYS	1422	
TTT	TGT	CAT	GCA	TCT	AAG	GAG	ATT	ACT	CCA	TTA	AAA	GAA	GGC	AAC	CCA	TGC	TGC	TGC	1422	
CYS	TGC	TGA																		

FIGURE 23C

